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Developing White Ash with Resistance to the Emerald Ash Borer

Kaitlin Joy Palla
Purdue University, kpj295@gmail.com

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DEVELOPING WHITE ASH WITH RESISTANCE TO THE EMERALD ASH BORER

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To my family and friends, for getting me through all rough patches with my sense of humor intact.

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CHAPTER 1. LITERATURE REVIEW

1.1 White ash

A member of Oleaceae (the olive family), white ash (*Fraxinus americana* L.) is one of 16 ash species native to North America. White ash joins green ash (*F. pennsylvanica*) and eight other species in the *Melioides* section; grouped together by molecular similarities and distinct from other species of *Fraxinus* by a strictly dioecious nature, flowers exhibiting a calyx, and samaras with terete seed cavities (Wallander 2008). White ash flowers are apetalous and form numerous small clusters, with a 1:1 ratio of male:female trees occurring in nature. Wind-pollinated, seed crops vary widely from year to year (Grisez 1975), with the minimum seed bearing age starting at 20 years. Pinnately compound, deciduous leaves are composed of five to seven opposite, ovate to ovate-lanceolate leaflets. Leaf scars form a distinctive C-shape below the leaf bud, a characteristic commonly used to distinguish *F. americana* from the similar-featured *F. pennsylvanica*, which exhibits D-shaped leaf scars (Brown-Rytlewski and Thompson 2003).

White ash is the largest and most common native ash, distributed across the eastern portion of North America. Ranging from Nova Scotia and Minnesota down to Texas and Florida, white ash reaches optimal growth on rich, moist, well-drained soils high in nitrogen, calcium, and magnesium (Schlesinger 1990; Royo and Knight 2012). Fast-

growing and shade-tolerant in its youth, white ash is a pioneer species and constant component in 26 forest cover types, particularly in the White Pine-Northern Red Oak-Red Maple type (Schlesinger 1990).

Fraxinus americana can be separated into three distinct ecotypes based on ploidy level and phenotypic variation. Cold-hardy, northern ecotypes are diploid (2n = 46), with bushy root systems and purple foliage in autumn. Winter-sensitive, southern types grow tap roots and exhibit diploid, tetraploid (2n = 92), or hexaploid (2n = 138) levels, as well as showy yellow leaves and red petioles in the fall. The third ecotype is an intermediate between these two, resembling the northern type phenotypically, but containing a polyploidy series (Wright 1944).

White ash is an ecologically important tree in North America. It is considered one of the best soil-improving species because the leaves are palatable to earthworms, allowing for its calcium-rich leaves to be recycled quickly (Johnston 1939). The trees are utilized as shelter and nesting sites by a variety of wildlife. The seeds are food for several bird and mammal species, and its bark was found to be utilized by rabbits, deer, beaver, cattle, and porcupine (Schlesinger 1990).

The vibrant fall color, relatively fast growth, and tolerance to a wide range of soil pH make *F. americana* horticulturally desirable as well. It has been planted extensively in urban areas and has been used to reclaim disturbed sites such as mines and abandoned farmland (Schlesinger 1990; Zeleznik and Skousen 1996; Nesom 2001). In 2005, ash trees composed 5% to 29% of all street trees in the Midwest, replacing many of the elm trees in urban settings since the advent of Dutch elm disease (MacFarlane and Meyer 2005). White ash cultivars tend to be sensitive to calcium and magnesium deficiencies, as

well as drought, particularly in urban environments where other stresses such as soil compaction, pollution, and salt build up were present (Percival et al. 2006; Royo and Knight 2012). Stressed trees were shown to be in particular danger of falling to disease and insect attack (Knight et al. 2012). There are several native borers that cause occasional damage to white ash (*Hylesinus aculeatus, Podosesia syringae, Tylonotus bimaculatus, Neoclytus acurninatus,* and *N. caprea*) (Schlesinger 1990; Atkinson 2012), although these only act as secondary pests on already weakened trees, unlike the recent invader, the emerald ash borer (*Agrilus planipennis* Fairmaire) that was able to attack healthy trees (Cappaert et al. 2005; Poland and McCullough 2006). Of the native borers, only the eastern ash bark beetle, *H. aculeatus* (Coleoptera: Curculionoidea), feeds exclusively on ash trees (Atkinson 2012).

White ash is the most economically valuable of the various ash species. As a commercial hardwood, its timber is valued for strength, straight grain, heavy weight, and elasticity. The wood is highly valued for the production of tool handles, furniture, flooring, crates, boats, doors, and cabinets. It is also the wood most often used in the production of baseball bats (Schlesinger 1990; Nesom 2001). In 2007, the estimated value of the 3.8 billion urban ash trees in the USA was $2.4 trillion, with an additional $282.3 billion in compensatory value from the 7.5 million ash trees growing on US timberlands (Sydnor et al. 2007).

1.2 Emerald ash borer

1.2.1 Biology and range

North American ash resources are currently being threatened with extinction by the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). EAB is an aggressive, wood-boring beetle indigenous to China, Japan, Taiwan, Korea, Mongolia, and eastern Russia (Yu 1992). Although only a minor pest of *Fraxinus* in its native area, EAB has become a devastating invasive pest to the North American landscape, particularly preferring to feed on white ash (Pureswaran and Poland 2009; Tanis and McCullough 2012).

Genetic analysis of EAB populations in North America showed that there was little diversity among the beetles here, likely descending from a single introduction or multiple introductions from similar source populations (Bray et al. 2011). Since being discovered in southeastern Michigan in 2002, the beetle has spread rapidly throughout at least 19 states (Michigan, Connecticut, Illinois, Indiana, Iowa, Kentucky, Maryland, Minnesota, Missouri, New Hampshire, New York, Ohio, Pennsylvania, Tennessee, Virginia, West Virginia, Kansas, Massachusetts, and Wisconsin) and parts of Canada (Ontario and Quebec) (Emerald Ash Borer Information Website 2013), largely because of the transport of infested ash material by humans, and the widespread planting of susceptible ash species in urban settings, where stressful conditions make trees susceptible to attack (Cappaert et al. 2005; Polland and McCullough 2006; Knight et al. 2012).

Adult beetles feed on the foliage of ash trees and bore D-shaped holes into the bark upon emergence in the spring, but the greatest damage is done by the larval stage. The

larvae bore through the cambial region, feeding on the nutrient-conducting phloem tissues and producing galleries that disrupt the flow of resources between the roots and leaves, girdling and eventually killing the tree. Over one-half of a tree's canopy can be devastated within the first 2 years, and even large trees may die within 3- to 4-years after first observed symptoms (McCullough and Katovich 2004). EAB is fatal to ash trees, and there is neither any known innate resistance in native trees nor any means to completely eradicate the beetle. Over 99% tree mortality in a stand was probable within 4 years of the first observed symptoms of infestation (Knight et al. 2012).

Unlike Asian ash species (*F. mandshurica* and *F. chinensis*), which exhibit some resistance to the beetle, all North American ash species are susceptible (Poland and McCullough 2006), although blue ash (*F. quadrangulata*) attracts lower densities of EAB (Pureswaran and Poland 2009; Tanis and McCullough 2012). Blue ash was found to have a distinct phenolic phloem chemistry profile disparate from other native ash species, which was thought to contribute to its relative resistance (Whitehall et al. 2012). Four differentially expressed proteins were identified in Manchurian ash that may play a role in EAB resistance (Whitehall et al. 2011). Resistance genes from these two species could provide assistance for biotechnology or traditional breeding programs working to introgress native ash defense genes into susceptible ash species. This is desirable, as there is evidence that EAB is spreading quickly throughout North America, placing ash trees in danger of being decimated (Dobesberger 2002).

1.2.2 EAB control options

The federal, state, and county quarantines in place around areas of detected infestation have slowed, but not limited, the spread of the beetle. Although transport of ash material

in these areas is regulated, mated EAB females fly an average of three miles in their lifetime, steadily expanding the range (Taylor et al. 2010). Eradication measures are costly and unreliable since they rely on detection of the beetle in order to be enacted (Polland and McCullough 2006). To date, there has been little impact being made on EAB populations from the introduced and indigenous parasitoids integral to the biological control program started by the United States Department of Agriculture (USDA) Forest Service and the Animal and Plant Health Inspection Service (APHIS) (Duan et al. 2012). Chemical controls using emamectin benzoate (TREE-äge™) and neonicotinoid insecticides such as imidacloprid and dinotefuran were shown to be extremely effective in controlling both adult and larval densities on trees (Poland et al. 2011). These controls, however, are expensive and inappropriate for protecting ash trees in forested or riparian areas (Bauer and Londoño 2011; Hahn et al. 2011), as well as environmentally undesirable (Poland and McCullough 2006). Such broad-spectrum insecticides can negatively affect non-target insects, causing ecological damage. Trunk-injected insecticides may also cause unintended harm. Annual applications were necessary to prevent EAB infestation, but repeated or improper drilling can result in stress and injury (Cappaert et al. 2005; Hahn et al. 2011). Surface application of microbial pesticides such as *Beauveria bassiana* strain GHA (Liu and Bauer 2008) or *Bacillus thuringiensis* strain SDS-502 (Bauer and Londoño 2011) have shown some success in EAB control, and were a less toxic option than synthetic chemical insecticides. The efficacy of these compounds was highly dependent on proper timing, environmental conditions, and dosage of application.

1.2.2.1 Bacillus thuringiensis

Bacillus thuringiensis (*Bt*) is a naturally occurring soil bacterium that produces proteinaceous crystals during sporulation. These crystals, comprised of proteins called delta-endotoxins, have potent insecticidal properties specific to a narrow taxonomic group of insects. The crystalline proteins (Cry toxins) are activated in the gut of insects, where the alkaline environment and presence of specific proteases cleave the toxin into its active form. The toxin then binds to receptors on the surface of the midgut, causing pores to form and fatally disrupting the movement of solutes. *Bt* is harmless to mammals, which have an acidic gut, and was damaging only to the insects that have the specific proteases and receptors necessary to bind to individual toxin strains (Schnepf et al. 1998). Around 100 subspecies of *Bt* have been classified, with relatively good correlation to the particular insect host range affected at the family level (summarized in Sanahuja et al. 2011).

Bt-based insecticides are created utilizing the toxin produced by the bacterium's spores. The specificity of these toxins' host range, as well as their lack of activity in mammals and biodegradable nature, makes *Bt* products considered some of the safest and most potent controls available. Detrimental off-target effects are limited. *Bt* at 100x field concentration rates had no effect on earthworms (Addison and Holmes 1996). As with chemical insecticides, topical applications of *Bt* were not efficient for forest settings or extensive areas, require proper application, and were subject to quick degradation (Poland et al. 2006). *Bt* was rapidly inactivated by UV light, heat, and extreme pH, and was easily displaced from the plant surface by wind or rain (summarized in Sanahuja et al. 2011). Laboratory bioassays have shown that *Bt* SDS-502, a strain of *Bacillus*

thuringiensis producing the Cry8Da toxin (Asano et al. 2003), to be virulent against EAB adults (Bauer and Lodoño 2011).

1.3 *Bt*-conferred insect resistance in forest trees

Genetically engineering trees to express *Bt* transgenes is an option that may be preferable to sprays. External control methods for EAB, such as insecticides and parasitoids, by their very nature fail at being significantly effective against the most damaging life stage of the beetle – the larval stage. Systemic insecticides can suffer from low or uneven uptake and also travel through the xylem, limiting the efficacy against phloem-feeding insects such as EAB (Poland et al. 2006). Since transgenic trees would produce the toxin within their tissues, phloem-feeding beetle larvae can be targeted. Internal control also eliminates environmental and non-target exposure resulting from external insecticide applications. The ability of genetically engineered trees to produce toxin continuously would avoid other issues associated with spray applications such as biological degradation, persistence, timing, and cost. The predicted cost of treating, removing, and replacing urban forest trees affected by EAB was expected to exceed $10 billion by 2019 (Kovacs et al. 2010). Genetically modifying ash trees to express resistance genes such as the *Bt Cry* genes is an important and desirable alternative for obtaining a cost-efficient, effective, and environmentally friendly means of controlling EAB in the North American landscape. Insect resistance through the expression of *Bt* toxin proteins directly in plant tissue was a strategy that has been shown to be extremely successful in crop plants (summarized in Sanahuja et al. 2011).

1.3.1 Transgenic *Bt* forest tree species

The first transgenic *Bt* tree was developed by McCown et al. (1991), where a *Cry1Aa* gene was introduced into a *Populus* hybrid. The transgenic plants exhibited a high level of resistance to both the forest tent caterpillar (*Malacosoma disstria*) and gypsy moth (*Lymantria dispar*) during subsequent leaf feeding assays (Kleiner et al. 1995).

Bt transgenes have since been successfully transferred into various species of *Eucalyptus* (Harcourt et al. 2000)*, Juglans* (Dandekar et al. 1998), *Larix* (Shin et al. 1994), *Malus* (James et al. 1993), *Picea* (Ellis et al. 1993; Lachance et al. 2007), *Pinus* (Tang and Tian 2003; Grace et al. 2005), and *Populus* (Robison et al. 1994; Balestrazzi et al. 1994; Wang et al. 1996; James et al. 1999; Meilan et al. 2000; Hu et al. 2001; Génissel et al. 2003; Kleiner et al. 2003) via biolistics or *Agrobacterium*-mediation transformation. Biolistic techniques such as particle bombardment were noted to be associated with potential instabilities or silencing of transgenes (Altpeter et al. 2005).

The level of transgene expression as well as tissue specificity are important factors in effective insect resistance. Ellis et al. (1993) noted few signs of spruce budworm toxcitity during feeding assays with *Picea glauca* callus cultures that had been transformed with the *Cry1Ab* gene. This was concluded to be a result of the low transgene expression in the embryonic tissue. Variable resistance in *Pinus radiata* plants containing a *Cry1Ac* gene was likewise observed. The age of transgenic needles was significant for the level of resistance shown; mature needles proved to be more toxic to painted apple moth (*Teia anartoides*) larvae feeding (Grace et al. 2005).

James et al. (1999) showed that the use of a vector containing a *Cry3A* gene under control of the cauliflower mosaic virus (CaMV) 35S promoter to transform *Populus*

resulted in highly effective resistance to the cottonwood leaf beetle (*Chrysomela tremulae*) damage among almost all transformants. The CaMV 35S promoter was subsequently used in conjunction with a synthetic *Cry3Aa* gene that had been modified to eliminate adenine and thymine-rich (AT-rich) regions and possess dicotyledonous codon usage, thereby optimizing the *Cry* gene for use in plant systems (Génissel et al. 2003). Bacterial genes have different codon preferences than plants, as well as lower guanine and cytosine content and frequent AT-rich regions. Modifications of *Cry* genes to be optimized for expression in plants increased toxin protein levels up to 100-fold (summarized in Sanahuja et al. 2011). Génissel et al. (2003) noted that transgenic *Populus* containing this *Cry3Aa* gene resulted in cottonwood leaf beetle mortality, even when feeding occurred on leaves with low-expression levels. Tang and Tian (2003) reported a nearly eight-fold increase in toxicity to larval feeding by transforming *Pinus* plants with a similar CaMV 35S-driven synthetic *Cry1Ac* gene.

Genetically transforming white ash to express a full length, codon-optimized *cry8Da* protoxin, optimized to plant transcriptional and translational machinery, and driven by a strong promoter such as the CaMV 35S, could be used in the development of EAB-resistant trees.

1.3.1.1 Insect resistance to *Bt*

Resistance is a concern for any insecticide because of selection pressures placed on target pests (Bauer 1995). *Bt* toxins mode of activation by specific receptors also increases the possibility for pests to evolve resistance. In laboratory experiments, *Bt*-resistant strains of mealmoths (*Plodia interpunctella*) formed after 15 generations of sublethal selection (McGaughey 1985). Other resistant varieties of insects were able to be

induced in the lab setting through intensive exposure to single strains of Cry protein. Resistance was also observed in wild populations of diamondback moth (*Plutella xylostella*) feeding on watercress after 400 spray periods (Liu and Tabashnik 1997).

The commercial agronomic crop industry has avoided this problem by using preventative measures like refuges (plantings of non-transgenic varieties) to maintain a non-resistant breeding population of target pests, even in high-intensity monoculture plantings. Enhancement of toxin activity by coexpression or protein fusion, as well as resistance pyramiding (combining different resistance genes) are further options for combatting resistance that can arise from continuous selection with only one toxin strain (summarized in Sanahuja et al. 2011).

1.3.1.2 Stability of transgenes

The long lives of forest trees, as well as the complexity of environments in which they grow, pose considerations for the durability of transgenes and the stability of expression. Investigations with annual crops have shown changes can occur seasonally as well as with a plant's age, and were widely reported (summarized in Ahuja 2009). Newly produced transgenic plants have also been shown to exhibit variability in expression. Most of the changes that occured were transcriptional or post-transcriptional gene silencing. Plant lines carrying fewer copies of the transgene were usually associated with stable expression. For transgenic trees, transgenes for introduced traits may be required to endure through both the vegetative and reproductive stages of growth. Changes in gene expression may potentially occur as a tree ages. Most studies that have been conducted were short-term, and expression was observed to be fairly stable. Field studies over 3 years conducted on *Populus* and *Picea* have also shown relatively stable expression of

transgenes conferring herbicide and insect resistance (Lachance et al. 2007; Li et al. 2008). Further long-term studies are necessary to evaluate continued stability. A number of studies have reported stable transgene expression for qualitative traits such as *Bt*-conferred insect resistance, as they were dominant exogenes, with gain-of-function (Hoenicka and Fladung 2006; Li et al. 2008). The type of promoter used may also affect expression. The CaMV 35S promoter regulates high transgene expression and appears to function well in most herbaceous and tree species (summarized in Ahuja 2009). Pons et al. (2012) reported stable long-term expression of two common marker genes (β-glucuronidase and neomycin phosphotransferase II) in transgenic citrus trees grown under agricultural conditions. Little seasonal variation was detected in expression levels over 3 years, which confirmed the absence of any rearrangements and silencing.

Stability of transgenes inducing reproductive sterility is particularly important for long-term genetic containment in trees. Trees disperse pollen and seed over long distances, and because of the limited cultivation of species, have a high potential for mating with wild relatives (James et al. 1998; Hoenicka and Fladung 2006). Transgene dispersal from genetically engineered trees into feral forest populations is a major biological and regulatory concern facing commercialization.

1.3.2 Regulation of transgenic trees

The genetic engineering of forest trees has been undertaken to confer a variety of desirable qualities such as pest and disease resistance, abiotic stress tolerance, improved wood properties, and flowering or yield traits (summarized in Harfouche et al. 2011). Development of genetically engineered trees has been constrained by the United States regulatory system, controlled by the USDA APHIS, the Food and Drug Administration

(FDA), and the Environmental Protection Agency (EPA). The primary concern with transgenic trees is their potential environmental risk. Deregulation of articles such as genetically engineered trees begins with obtaining a permit for release into the environment. Once approved, field testing is done to provide information about the regulated item and to ensure that the plants are as safe to use as those generated by traditional breeding. A petition to deregulate the article is submitted to APHIS for evaluation after field trials have been conducted. APHIS determines whether or not deregulation will occur, and has the option to place restrictions on the article based on their assessment.

Thus far, only two transgenic trees have been deregulated in the United States, the papaya and the plum tree, although field trials for a number of transgenic tree species have been conducted over the past 20 years. Field trials are terminated prior to the onset of flowering to ensure no transgene escape can occur (summarized in FAO 2010; Viswanath et al. 2012). *Bt* transgenic poplars released and outplanted in China in 2001 have significantly inhibited the otherwise rapid spread of target pests in commercial plantations. Necessary insecticide treatments were also reduced by the presence of transgenic trees. Transgenic *Populus nigra* has been employed as an insect-resistant source of genetic material in recent breeding of hybrid clones (Lu and Hu 2011). The benefit of mixed transgenic and non-transgenic plantings has been shown in commercial papaya, where trees transformed to be resistant to the papaya ringspot virus kept infestation rates low enough to allow the production of non-resistant varieties (Walter et al. 2010).

Research in creating genetically modified trees has only been increasing, outpacing the biosafety and scientific assessments of their possible ecological impact, and resulting in an increase of restrictions before full deregulation (Strauss et al. 2010). The main concern with transforming perennial plants such as trees is the ecological impact transgenes could have by escaping into feral populations or through conferring a selection advantage. With the threat of EAB so widespread and dire, it can be argued that resistance transgene escape would be beneficial in repopulating native ash resources and reducing the beetle's populations. Recently, the transport of the Cry1Ac protein was observed between transgenic poplar rootstock and non-transgenic scion (Wang et al. 2012). This protein transport occurred mainly through the phloem and migrated in high enough concentrations that the non-transgenic leaves had a lethal effect on *Clostera anachoreta* larvae during laboratory feeding assays. The use of transgenic white ash as root stock to confer resistance to EAB could be a possibility that negates pollen dispersal concerns, solving some regulatory issues.

1.4 *Fraxinus* spp. in vitro

The first attempt to establish in vitro *Fraxinus* cultures (Gautheret 1934) failed to generate either callus or shoots. It was not until several years later that callus cultures of *F. pennsylvanica* and *F. americana* exhibiting continual growth were obtained by Wolter and Skoog (1966) and Hicks and Browne (1983), respectively. Since these initial efforts, in vitro propagation of *Fraxinus* spp. through axillary shoot micropropagation, somatic embryogenesis, and adventitious shoot organogenesis has been reported for several of the most valued species.

1.4.1 Micropropagation of *Fraxinus* spp.

The first limited development of *Fraxinus* buds in vitro was reported by Browne and Hicks (1983), and while long term growth was not achieved, the promotive effect of benzyladenine (BA) was noted. Since then, the successful micropropagation of shoots in vitro has been reported for *F. americana, F. angustifolia* (narrow-leaf ash), *F. excelsior* (common ash), *F. ornus* (flowering ash), and *F. pennsylvanica* (summarized in Van Sambeek and Preece 2007; Mitras et al. 2009), as well as for *F. mandshurica* (Manchurian ash) (Zhang et al. 2007) and *F. micrantha* (native to Himalaya) (Bisht et al. 2011). Surface disinfestation with dilute solutions of disinfectants such as ethanol and sodium hypochlorite (NaOCl) was consistently observed to be successful in decontaminating explants for culture establishment.

The growth stage of the plant tissues used as well as the choice of medium have the most significant effects on successfully obtaining clean, proliferating *Fraxinus* spp. cultures. Shoot tips and nodal segments from mature or grafted material were often difficult to establish in vitro, suffering from high contamination rates and limited axillary shoot growth, although some success was reported for several ash species (Preece et al. 1987; Arrillaga et al. 1992a; Hammatt 1994; Perez-Parron et al. 1994; Schoenweiss and Meier-Dinkel 2005). Juvenile material is much easier to disinfest than mature explants, and is more amenable to the establishment and proliferation of axillary shoots. Shoot tip and nodal explants, as well as seed explants have been used with success, particularly the vegetative buds from zygotic embryos (Preece et al. 1987, 1995; Navarrete et al. 1989; Chalupa 1990; Hammatt and Ridout 1992; Silveira and Cottignies 1994; Van Sambeek et al. 2001; Mitras et al. 2009; Bisht et al. 2011). In vitro germination of seeds has been

shown to benefit from cutting a portion of the seed, opposite the radicle. This promotive effect was also noted for excised embryos whose cotyledonary tips had been removed (Preece et al. 1989, 1995; Arrillaga et al. 1992b; Van Sambeek et al. 2001; Ashley and Preece 2009).

Agar-solidified, high-salt basal medium with the pH adjusted to around 5.7 produced the most success in establishing and proliferating *Fraxinus* spp. axillary shoot cultures. Murashige and Skoog (1962) (MS) and Driver and Kuniyuki (1984) (DKW) media were common choices, particularly for *F. americana* and *F. pennsylvanica* (summarized in Van Sambeek and Preece 2007). Woody plant medium (WPM) (Lloyd and McCown 1981) was reported successful for micropropagation of *F. excelsior, F. mandshurica,* and *F. micrantha* (Silveira and Cottignies 1994; Zhang et al. 2007; Mitras et al. 2009; Bisht et al. 2011), and Quoirin and Lepoivre (QL) medium (Quoirin and Lepoivre 1977) for micropropagation of *F. angustifolia* and *F. ornus* (Perez-Parron et al. 1994; Leforestier et al. 1991; Arrillaga et al. 1992a). Elongation of epicotyls and axillary shoot growth from germinating embryos was accelerated by the addition of a liquid medium overlay during culture on agar solidified medium (Preece et al. 1995; Arrillaga et al. 1992a; Van Sambeek et al. 2001).

Germination and axillary shoot proliferation was stimulated by the addition of various plant growth regulators (PGRs) to the basal medium, namely thidiazuron (TDZ), BA, and indole-3-butyric acid (IBA). High concentrations of BA, either alone or in combination with TDZ and low concentrations of IBA were usually the most successful for ash micropropagation.

1.4.2 Adventitious rooting of *Fraxinus* spp.

Adventitious rooting has been reported for *F. americana, F. angustifolia*, *F. excelsior*, *F. ornus*, and *F. pennsylvanica* (summarized in Van Sambeek and Preece 2007; Du and Pijut 2008; Mitras et al. 2009; Palla and Pijut 2011), as well as for *F. mandshurica* (Tan and Shen 2003; Zhang and Luo 2003), *F. micrantha* (Bisht et al. 2011), *F. nigra* (reported in Pijut et al. 2011), and *F. profunda* (pumpkin ash) (Stevens and Pijut 2012). Ex vitro rooting has been achieved for green and white ash under mist in the greenhouse, both with and without the use of a quick dip in 1mM IBA (summarized in Van Sambeek and Preece 2007), although in vitro methods were more often used.

In vitro root development of *Fraxinus* spp. was typically initiated with a short pulse on medium containing auxin, followed by culture on PGR-free medium to elongate roots. Rooting was favored by low-salt medium. Half- or full-strength WPM supplemented with IBA with or without naphthaleneacetic acid (NAA) was most often used for inducing root formation, although MS medium with B5 vitamins (Gamborg et al. 1968) (MSB5) (Kim et al. 1998) and MS medium have also been used successfully (Preece et al. 1987; Navarrete et al. 1989; Van Sambeek et al. 2001; Tan and Shen 2003). There have been reports of *Fraxinus* spp. rooting on auxin-free medium, but it was noted that the addition of auxin provided more synchronous root formation (Preece et al. 1995; Kim et al. 1998; Tonon et al. 2001d; Bisht et al. 2011; Stevens and Pijut 2012). *Fraxinus americana*, *F. angustifolia*, *F. pennsylvanica*, and *F. profunda* root induction benefited from a 4- to 10-day dark pulse prior to placing cultures under a 16 hour photoperiod to elongate roots (summarized in Van Sambeek and Preece 2007; Du and Pijut 2008; Palla and Pijut 2011; Stevens and Pijut 2012). The most recent adventitious rooting protocol for white ash

achieved high rooting rates (81%) on WPM supplemented with 4.9 µM IBA and 2.9 µM indole-3-acetic acid (IAA). This improved upon previous reports by requiring one single medium formulation in conjunction with a 5-day dark pulse (Palla and Pijut 2011).

1.4.3 Adventitious shoot regeneration of *Fraxinus* spp.

In order to develop trees exhibiting a trait of interest, such as insect resistance, it is necessary to establish a genetic transformation system that will allow for the integration of specific genes. The first step in creating such a protocol is to establish an efficient method of regenerating plants in vitro. Adventitious shoot regeneration can occur either by somatic embryogenesis or shoot organogenesis.

1.4.3.1 Somatic embryogenesis of *Fraxinus*

Somatic embryogenesis has been reported for *F. americana* (summarized in Preece and Bates 1995), *F. angustifolia* (Tonon et al. 2001a, 2001c), *F. chinenses* subsp. *rhynchophylla* (Chinese ash) (Yang et al. 2012), *F. excelsior* (Capuana et al. 2007), *F. mandshurica* (Kong et al. 2006, 2012), and *F. micrantha* (Bisht et al. 2011). Of the six species, *F. americana* initiated the best embryogenesis from mature zygotic embryos. Immature zygotic embryos were the ideal explants to use in producing somatic embryos for *F. angustifolia, F. chinensis* subsp. *rhynchophylla*, *F. excelsior*, *F. mandshurica*, and *F. micrantha*. All species responded positively to BA, proving it to be beneficial for somatic embryogenesis, usually in conjunction with 2,4-dichlorophenoxyacetic acid (2,4-D). It was also noted that TDZ increased somatic embryogeneis for *F. americana* and *F. mandshurica* (Bates et al. 1992; Kong et al. 2012). The conversion of somatic embryos into plantlets was achieved, although the rate of conversion varied by species (summarized in Preece and Bates 1995; Tonon et al. 2001c; Capuana et al. 2007; Bisht et

al. 2011; Kong et al. 2012; Yang et al. 2012). Preece and Bates (1995) observed a definite genotypic effect on *F. americana* embryogenesis potential, and were able to stimulate no more than 20% of seeds to form somatic embryos. The low plantlet conversion rates make current somatic embryogenesis protocols unsuitable to use in a genetic transformation system for white ash.

1.4.3.2 Shoot organogenesis in *Fraxinus* spp.

Adventitious shoot regeneration through organogenesis has been achieved for several species of *Fraxinus* with various explant sources. Bates et al. (1992) noted adventitious bud and shoot formation from callus, as well as directly on the cotyledons and hypocotyls from mature white ash seeds. Kim et al. (1997) and Van Sambeek et al. (2001) also reported organogenic callus forming on the cut surfaces (cotyledon, hypocotyl, and shoot base) of *F. americana* and *F. pennsylvanica* tissue in contact with the culture medium, resulting in adventitious shoots. Hammatt (1994, 1996) found *F. excelsior* leaf explants, petioles, and cotyledons were able to form adventitious shoot buds. However, only a few shoots in these studies elongated enough to be rooted. It is interesting to note that in all cases adventitious shoots formed when the cut portion of the explant was exposed to medium supplemented with 4.4 μM or 10 μM TDZ.

Reports of regeneration from cotyledons and hypocotyls of *F. angustifolia* (Tonon et al. 2001b), *F. pennsylvanica* (Du and Pijut 2008), and *F. americana* (Palla and Pijut 2011) all noted a lower organogenic potential of cotyledons compared to hypocotyls. Hypocotyls have also been used successfully for regenerating adventitious shoots of *F. excelsior* (Tabrett and Hammatt 1992; Capuana et al. 2007), *F. mandshurica* (Tan and

Shen 2003), *F. micrantha* (Bisht et al. 2011), *F. profunda* (Stevens and Pijut 2012), and *F. nigra* (reported in Pijut et al. 2011).

MS medium was predominantly used for *Fraxinus* spp. adventitious shoot regeneration (summarized in Van Sambeek and Preece 2007; Tan and Shen 2003; Du and Pijut 2008; Palla and Pijut 2011; Stevens and Pijut 2012). Hammatt (1994, 1996), Tabrett and Hammatt (1992), and Tonon et al. (2001b) found transferring explants to DKW medium after shoot induction was beneficial for elongation of shoots. However, Du and Pijut (2008), Palla and Pijut (2011), and Stevens and Pijut (2012) achieved high shoot elongation and proliferation rates by transferring explants to MSB5 medium supplemented with 10 μM BA and 10 μM TDZ after induction. The presence of TDZ at any concentration proved effective in inducing adventitious bud regeneration (summarized in Van Sambeek and Preece 2007; Tan and Shen 2003; Du and Pijut 2008; Palla and Pijut 2011; Stevens and Pijut 2012), although extended cultivation with this PGR was found to be deleterious in some instances (Tabrett and Hammatt 1992). BA, particularly in higher concentrations (up to 22.2 μM), proved beneficial for regenerating, elongating, and proliferating adventitious shoots for all species of *Fraxinus*. In fact, Tonon et al. (2001b) noted that constant exposure of *F. angustifolia* explants to BA was essential for regeneration.

The white ash adventitious shoot regeneration and rooting protocol described by Palla and Pijut (2011) was efficient, requiring only a single medium formulation for each step of shoot induction, shoot elongation, and adventitious rooting. No additional medium overlays or auxin rooting dip treatments were necessary to obtain complete plantlets, as had previously been reported (summarized in Van Sambeek and Preece 2007).

Hypocotyls were amenable to genetic transformation systems, as it was observed that wounding stimulated adventitious shoot regeneration (Tonon et al. 2001b). Palla and Pijut (2011) provide a method for producing entire plants from single explants that will be helpful in developing a genetic transformation system for white ash.

1.4.4 Genetic transformation of *Fraxinus* spp.

The first attempt at genetic transformation of ash was by Roome (1992), where *F. pennsylvanica* callus was obtained after culture on selection medium containing the antibiotic kanamycin. The callus was confirmed to be transformed through polymerase chain reaction (PCR) and Southern blot analysis, but no stably transformed shoots were ever regenerated. White ash transformation was attempted by Bates (1997), but while multiple shoots were produced in vitro after *Agrobacterium* infection, none of these were confirmed to be transgenic. Bates (1997) did show that infection of white ash with *Agrobacterium* was possible. Adventitious shoot regeneration and rooting protocols previously developed for *F. pennsylvanica* (Du and Pijut 2008) have been successfully used as a framework for *Agrobacterium*-mediated genetic transformation (Du and Pijut 2009). Three transgenic plantlets were produced containing the neomycin phosphotransferase (*nptII*) and β-glucuronidase (GUS) fusion gene, and an enhanced green fluorescent protein gene. The presence of the GUS and *nptII* genes were confirmed by PCR and Southern blotting. Du (2008) further reported several transgenic green ash callus cultures, confirmed through PCR, containing a *Cry8D2* gene. No transgenic shoots were produced, but a precedent for transforming ash to express a *Bt* toxin gene was established. The *Cry8D2* gene used was optimized for the plant transcriptional and translational machinery by increasing the GC content from the naturally occurring *Bt*

sequence without altering the peptide sequence. The Cry8Da toxin was produced by *Bt* SDS-502 (Asano et al. 2003), a strain of *Bacillus thuringiensis* shown to be virulent against EAB adults (Bauer and Lodoño 2011). With a thought to the concern of transgene escape, an *AGAMOUS* gene homolog (*FpAG1*) was cloned and characterized from green ash (Du and Pijut 2010). The disruption of this gene could potentially help in developing sterile *F. pennsylvanica* trees, preventing gene flow from any transgenics released.

The success of translating the Du and Pijut (2008) green ash regeneration system into a transformation system (Du 2008; Du and Pijut 2009), as well as the earlier reports of *F. nigra* (reported in Pijut et al. 2011) and *F. profunda* (Stevens 2012) transformation using previous regeneration protocols holds promise that the white ash regeneration protocol of Palla and Pijut (2011) can be used to develop transgenic *F. americana* plants. A reliable transformation system for white ash would allow the opportunity to integrate useful traits such as insect resistance. One of the objectives of this project was to produce white ash plants expressing a *Bt* toxin gene (*Cry8Da*) in the hopes of conferring resistance to EAB. This is becoming increasingly desirable as the threat posed by the exotic emerald ash borer becomes more desperate.

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CHAPTER 2. REGENERATION OF PLANTS FROM *FRAXINUS AMERICANA* HYPOCOTYLS AND COTYLEDONS

Palla KJ, Pijut PM (2011) Regeneration of plants from *Fraxinus americana* hypocotyls and cotyledons. In Vitro Cell Dev Biol Plant 47:250-256

2.1 Abstract

A plant regeneration protocol was developed for white ash (*Fraxinus americana* L.). Hypocotyls and cotyledons excised from embryos were cultured on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BA) plus thidiazuron (TDZ), and compared for organogenic potential. Sixty-six percent of hypocotyl segments and 10.4% of cotyledon segments produced adventitious shoots, with a mean number of adventitious shoots per explant of 3.5 ± 0.9 and 2.5 ± 1.5, respectively. The best regeneration medium (52% shoot formation; 47% shoot elongation) for hypocotyls was MS basal medium containing 22.2 μM BA plus 0.5 μM TDZ, producing a mean of 3.9 ± 0.4 adventitious shoots. Adventitious shoots were established as proliferating shoot cultures following transfer to MS medium with Gamborg B5 vitamins supplemented with 10 μM BA plus 10 μM TDZ. For in vitro rooting, woody plant medium with indole-3-acetic acid (IAA) at 0, 2.9, 5.7, or 8.6 μM in combination with 4.9 μM indole-3-butyric acid (IBA) was tested for a 5- or 10-d dark culture period, followed by culture under a

16-h photoperiod. The best rooting (78% to 81%) of in vitro shoots was obtained with a 5-d dark culture treatment on medium containing 2.9 or 5.7 μM IAA plus 4.9 μM IBA, with an average of 2.6 ± 0.4 roots per shoot. Rooted plants were successfully acclimatized to the greenhouse. This adventitious shoot regeneration and rooting protocol will be used as the basis for experimental studies to produce transgenic white ash with resistance to the emerald ash borer.

2.2 Introduction

White ash, *Fraxinus americana* L. (family Oleaceae), is one of the most abundant and useful of the native ash species, providing both ecological and economic benefits. Endemic to North America, this species is integral to many ecosystems across the east from Nova Scotia to Texas and was recognized as a component species in 26 forest cover types (Schlesinger 1990). White ash trees provide shelter and nesting sites for a variety of wildlife. The seeds are food for several bird and mammal species, and its bark was found to be utilized by rabbits, deer, beaver, and porcupine (Griffith 1991). White ash is valued as an ornamental shade tree and as a pioneer species, making it ideal for use in urban areas as well as reclamation of disturbed sites (Nesom 2001). Ash trees composed 5% to 29% of all street trees in the Midwest, replacing many of the elm trees in urban settings since the advent of Dutch elm disease (MacFarlane and Meyer 2005). White ash has high economic value as a commercial hardwood, with timber trees valued for wood strength, straight grain, heavy weight, and elasticity. The wood is used in the production of tool handles, furniture, flooring, crates, boats, doors, and cabinets. White ash is the wood most often used in the production of baseball bats. The estimated value of the 3.8 billion urban

ash trees in the USA was $2.4 trillion, with an additional $282.3 billion in compensatory value from the 7.5 million ash trees growing on US timberlands (Sydnor et al. 2007).

There exists a threat to white ash in the form of the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). EAB is an aggressive, wood-boring beetle indigenous to China, Japan, Taiwan, Korea, Mongolia, and eastern Russia (Yu 1992). EAB has become a devastating invasive pest to the North American landscape since being discovered in southeastern Michigan in 2002. The adult beetles feed on the foliage of ash trees, but most of the damage is produced by the larval stage. The larvae bore through the bark and into the cambial region, feeding and producing galleries that disrupt the flow of water and nutrients, eventually killing the tree. Over one-half of a tree's canopy can be devastated within the first 2-yr, and the entire tree will usually be dead within 3- to 4-yr after first observed symptoms (McCullough and Katovich 2004). EAB is fatal to ash trees, and there is neither any known innate resistance in native trees nor any means to completely eradicate the beetle (Poland and McCullough 2006). All native North American ash species are susceptible, and there is evidence that EAB is spreading quickly throughout North America, placing ash trees in danger (Dobesberger 2002). Devastation of this tree species as a result of EAB infestation will be a huge economic and ecological loss. Development of an in vitro protocol for regenerating white ash plants will prove valuable in producing improved ash germplasm. Such a protocol will also be necessary to genetically modify white ash to produce resistance to the EAB, thus benefiting economic markets and ecological landscapes.

Adventitious shoot regeneration has been attempted for several ash species. Callus formation was induced using isolated petioles of *F*. a*mericana*, but no shoot regeneration

from that callus was achieved (Browne and Hicks 1983). Navarrete et al. (1989) reported explant growth and axillary shoot proliferation from in vitro germinated cut white ash seeds. Bates et al. (1992) using cotyledons, hypocotyls, and epicotyls from mature white ash seeds regenerated adventitious buds and shoots, but only a few shoots elongated enough to be rooted. Adventitious shoot regeneration from hypocotyls (Tabrett and Hammatt 1992), and direct organogenesis from hypocotyls, embryonic leaves, and stems (Mockeliunaite and Kuusiene 2004) of common ash (*F. excelsior*) has also been reported. High rooting (76%) was achieved by Tonon et al. (2001b) for narrowleaf ash (*F. angustifolia*) by culturing shoots in the dark for 5 d on root induction medium then 15 d in the light on root expression medium. Du and Pijut (2008) developed a complete plant regeneration protocol using hypocotyls and cotyledons of green ash (*F. pennsylvanica*). Mitras et al. (2009) successfully used epicotyls for in vitro propagation of *F. excelsior*. The present study was initiated to develop a protocol for adventitious shoot regeneration and rooting of white ash for further use in genetic transformation studies.

2.3 Materials and Methods

2.3.1 Adventitious shoot induction

Mature white ash seeds purchased in 2004 from F.W. Schumacher Co., Inc. (Sandwich, MA) were stored in a sealed container in the dark at 5°C until used. The pericarps were cut and removed along with 2- to 3-mm of the seed opposite the radical. Any seeds with noticeable insect or fungal damage were discarded. Seeds were surface disinfested in 70% (v/v) ethanol for 30 s, then immersed in 20% bleach solution (5.25% sodium

hypochlorite) for 18 min, followed by three rinses in sterile, deionized water; before being stored in sterile, deionized water overnight at 24 ± 2°C. After 24 h, the turgid embryos were extracted and cotyledon and hypocotyl segments were cultured horizontally on Murashige and Skoog (MS) medium (1962) supplemented with 13.3 µM 6-benzylaminopurine (BA) plus 4.5 µM thidiazuron (TDZ) to induce adventitious shoot formation (Du and Pijut 2008). Four to five replications, of each explant type were tested, with 12 to 17 explants per replication. Unless noted otherwise, all cultures were incubated at 24 ± 2°C under a 16-h photoperiod (80 µmol m^{-2} s^{-1}), and all media included 3% (w/v) sucrose, 0.7% (w/v) Difco-Bacto agar, with the pH of the medium adjusted to 5.7 prior to autoclaving and dispensing at 45 ml per 100 mm x 25 mm Petri plates. After obtaining these results, adventitious shoot regeneration was optimized for hypocotyls using 0, 4.4, 8.9, 13.3, or 22.2 µM BA in combination with 0, 0.5, 2.3, or 4.5 µM TDZ (Du and Pijut 2008). Five replications with 15 hypocotyls per treatment per replication were conducted. Cultures were incubated for 4 wk to induce callus and shoot bud formation.

2.3.2 Elongation of adventitious shoots

After 4 wk culture on induction medium, all explants initiating shoot buds were transferred to MS basal salt medium plus Gamborg B5 vitamins (MSB5) supplemented with 10 µM BA and 10 µM TDZ (Kim et al. 1997) to induce shoot elongation. Magenta GA-7 vessels (Magenta Corp., Chicago, IL) containing 50 ml of medium were used. Cultures were incubated under a 16-h photoperiod for 4 to 5 wk. Elongated shoots were continuously removed from the original explant when approximately 3 cm in length, and placed directly onto root induction medium.

2.3.3 Establishment of in vitro germinated shoots

To supplement the number of adventitious shoots in culture for rooting experiments, proliferating shoot cultures were established from in vitro germinated embryos, in order to obtain a significant number of microshoots. White ash seeds were surface disinfected and the embryos extracted as described previously. Intact embryos were then cultured vertically on MSB5 medium supplemented with 10 µM BA plus 10 µM TDZ. Magenta GA-7 vessels containing 50 ml of medium were used. The in vitro shoots established from the embryos were micropropagated on the same medium, and continuously subcultured to fresh medium every 4 wk for shoot culture establishment.

2.3.4 Rooting of adventitious and in vitro germinated shoots

Elongated adventitious and in vitro germinated shoots were pooled and randomly placed on woody plant medium (WPM) (Lloyd and McCown 1980) supplemented with 4.9 µM indole-3-butyric acid (IBA) plus 0, 2.9, 5.7, or 8.6 µM indole-3-acetic acid (IAA). Magenta GA-7 vessels containing 50 ml of medium were used. Cultures were placed in the dark at 26°C for either 5- or 10-d, then incubated for 4- to 5-wk at 24 ± 2°C under a 16-h photoperiod, after which rooting percentage, the number of roots per shoot, and root length were recorded. Nine replications with four shoots per treatment per replication were conducted.

2.3.5 Acclimatization of rooted plants

Rooted plantlets were removed from the Magenta vessels and transplanted into 10 cm x 9 cm plastic pots containing a moist, autoclaved, soilless medium with high porosity (Premier ProMix HP; Premier Horticulture Inc., Quakertown, PA). Any agar clinging to the roots was gently removed with distilled water prior to potting. Plants in pots were

then placed in sealed 3.8 L plastic bags to provide a high relative humidity, and grown at 24 ± 2°C under a 16-h photoperiod. Watered as needed to maintain moisture, the plants were acclimatized to the culture room over a 2- to 3-wk period by progressively opening the bags until plants were able to survive without wilting. Plants were then transferred to the greenhouse, transplanted to larger pots containing a peat and perlite based, soilless medium with high porosity (Premier ProMix HP/ Mycorise® Pro; Premier Horticulture Inc., Quakertown, PA), and watered, as needed, with fertilizer water [15N-5P-15K commercial fertilizer formulation (Miracle Gro© Excel© Cal-Mag; The Scotts Co., Marysville, OH)] that was adjusted to a pH range of 5.7 to 6.0 using 93% sulfuric acid (Ulrich Chemical, Indianapolis, IN). Three replications of 12 plants per replication were acclimatized.

2.3.6 Statistical analysis

SPSS (Software Version 17) (SPSS 2009) was used to analyze data. An analysis of variance (ANOVA) was performed using the General Linear Model procedure on the individual replicate means by treatment for percent shoot formation, number of shoots per hypocotyl, percent root formation, number of roots per shoot, root length, and number of lateral roots. If the ANOVA indicated a statistical significance, a Duncan's comparison test with an alpha level of 0.05 was used to distinguish the differences between treatments.

2.4 Results and Discussion

2.4.1 Adventitious shoot induction

A collection of open-pollinated seed was used to normalize the regeneration process across a random mix of white ash genotypes. The tips of the cotyledons were removed during the surface disinfestation process to not only make it easier to excise the embryos post-imbibition, but because cutting one-third of the cotyledons had been proven to increase germination rates (Preece et al. 1989, 1995). After 1 wk culture on MS medium supplemented with BA and TDZ, callus formation occurred on the cut ends and wounded surface area of hypocotyls, and shoots began to initiate. Cotyledon explants took longer to form callus with many of these explants never regenerating shoots (Fig. 2.1). Both types of explants regenerated most shoots at the cut end previously nearest the shoot apical meristem.

Green and white ash are in the Melioides clade, and therefore have high nuclear and morphological similarity (Wallander 2008). The adventitious shoot regeneration protocol developed in our lab for green ash (*F. pennsylvanica*) (Du and Pijut 2008) was therefore used initially to compare the response of white ash hypocotyls and cotyledons, and determine the best explant for further development of the regeneration protocol. Hypocotyls proved to have a significantly higher organogenic potential than cotyledons in white ash (Fig. 2.2a; Table 2.1). Sixty-six percent of hypocotyl segments produced adventitious shoots, but only 10.4% of cotyledon segments regenerated in this way, with a mean number of adventitious shoots per explant of 3.5 ± 0.9 and 2.5 ± 1.5, respectively. This was consistent with results reported by Tonon et al. (2001a) who noted a lower

organogenic potential of *F. angustifolia* cotyledons compared to embryo axes. The lower shoot formation rate observed for white ash cotyledons (10.4%) compared to green ash cotyledons (24%) (Du and Pijut 2008) could be a result of white ash having a stronger and more complicated hormonally-controlled dormancy than green ash (Ashley and Preece 2009).

All combinations of BA and TDZ in the induction medium produced some level of adventitious shoot formation on hypocotyls. Seventy-three percent of hypocotyls produced shoots on medium containing only 0.5 μM TDZ, but this percentage did not accurately represent the best regeneration medium, as only 19% of the shoots elongated (Table 2.2). To better elucidate which medium would produce a high percentage of shoot formation along with shoot elongation, we present the percentage of hypocotyls producing shoots, percent shoot elongation, and mean number of shoots elongating per explant (Fig. 2.2b, c; Table 2.2). This was a more accurate representation of what medium was the most successful to use for white ash regeneration. Medium supplemented with 22.2 μM BA plus 0.5 μM TDZ proved to be the best adventitious shoot induction medium overall. Even though the percent shoot formation (52%) appears low, this medium produced the highest percentage (47%) of shoots able to elongate and the highest mean number of shoots elongating per hypocotyl (3.9 ± 0.4). The BA concentration was found to be a significant factor in determining the number of shoots initiated that would eventually elongate from the hypocotyl explants. This same BA concentration (22.2 μM) also proved successful for proliferation of adventitious shoots from *F. excelsior* hypocotyls (Tabrett and Hammatt 1992). The concentration of TDZ at 0.5, 2.3, or 4.5 μM was a significant factor in percent shoot formation on hypocotyls in

combination with zero BA, inducing a high percentage of shoot formation of 73, 63, and 69 %, respectively. This was consistent with the findings of Navarrete et al. (1989), who noted that 3 µM TDZ was the maximum concentration allowing for the most shoot proliferation of white ash while having minimal callus formation. With our protocol, only a 3- to 4-wk culture on one medium was necessary to induce shoots on hypocotyl explants. No additional plant growth regulator overlays or different medium transfers were necessary, as had previously been reported (Navarrete et al. 1989, Preece et al. 1991, VanSambeek et al. 2001).

2.4.2 Rooting of adventitious and in vitro grown shoots

A collection of in vitro shoots (adventitious and in vitro micropropagated) were randomly divided among eight root induction treatments. Roots were visible 10 d after transferring cultures to the light with slight formation of callus at the basal end of the shoot. Rarely did shoots form roots without a small amount of light green callus. Significantly higher rooting (53 to 81%), number of roots (1.8 to 3.6), and root lengths (2.7 to 4.1 cm) were obtained with the 5-d dark culture treatment. The most effective root induction medium was WPM supplemented with either 2.9 µM or 5.7 µM IAA plus 4.9 µM IBA for 5 d in the dark (Fig. 2.2d) with 81% and 78% of shoots rooted with an average of 2.5 ± 0.3 and 2.6 ± 0.4 roots produced per shoot, respectively. The number of lateral roots formed was not statistically significant across all treatments (Table 2.3). Preece et al. (1995), Kim et al. (1998), and Tonon et al. (2001a, b) reported rooting of *Fraxinus* spp. on auxin-free medium, but it was noted that the addition of auxin provided more synchronous root formation. Half- or full-strength WPM supplemented with 4.9 µM IBA had previously been proven successful for inducing root formation in *Fraxinus* spp.

(Hammatt and Ridout 1992; Tabrett and Hammatt 1992; Perez-Parron et al. 1994; Tonon et al. 2001a; Du and Pijut 2008) as well as MS and MSB5 medium (Preece et al. 1987; Navarrete et al. 1989; Kim et al. 1998). The addition of 2.9 µM to 8.6 µM IAA to WPM supplemented with 4.9 µM IBA produced significant rooting (89-90%) of *F. pennsylvanica* shoots (Du and Pijut 2008), along with a 10 d-dark pretreatment. Tonon et al. (2001b) achieved high rooting (76%) for *F. angustifolia* by exposing shoots to a root induction medium for 5 d in the dark before transferring shoots to a root expression medium, and then placing cultures in the light. A similar two-step process was used for *F. americana* and *F. pennsylvanica* (Navarrete et al. 1989, Van Sambeek et al. 2001) with a 4- to 8-d dark pulse on root induction medium followed by culture in the light on root elongation medium. Our rooting protocol eliminates the need to use two different media for root induction and elongation, and required only 5 d in the dark. We achieved 81% rooting of white ash shoots with an average of 2.5 roots per shoot, with a mean root length of 3.6 cm, and a mean number of lateral roots of 9.1 using one medium. Previously, approximately 70% of shoots rooted with an average of 2.2 roots per shoot after 7 d in the dark on root induction medium and culture in the light on root elongation medium (Van Sambeek and Preece 2001).

2.4.3 Acclimatization of rooted plants

Thirty-eight rooted plantlets were chosen randomly for acclimatization. Plantlets with two to three internodes, four to six fully formed leaves, and well developed roots were used (Fig. 2.2d). Simple leaves were observed on plantlets regenerated in vitro and acclimatized to the culture room (Fig. 2.2e), with compound leaf development observed after continued growth and acclimatization to the greenhouse (Fig 2.2f). This was

consistent with previous observations on growth of white ash started in vitro (Van Sambeek and Preece 2001). No morphological abnormalities were observed with our white ash plants, and 100% of the plants survived acclimatization to the greenhouse.

2.5 Conclusions

A successful in vitro adventitious shoot regeneration, rooting, and plantlet acclimatization protocol for *Fraxinus americana* was developed using hypocotyls obtained from a collection of open-pollinated seeds. This protocol will be used for experimental studies to produce transgenic white ash with resistance to the EAB, or transfer of other value-added traits for genetic improvement and conservation.

Table 2.1 Effect of explant type on Fraxinus americana adventitious shoot regeneration.

Explant Type	Callus Formation[a] (%)	Shoot Formation[a] (%)	Mean No. Shoot per Explant[a]
Cotyledon	28.9 ± 25.2	10.4 ± 8.0	2.5 ± 1.5
Hypocotyl	91.5 ± 6.4	66.1 ± 19.5	3.5 ± 0.9

[a]Values are means ± standard deviation of responsive explants (49 cotyledons; 72 hypocotyls) on Murashige and Skoog medium supplemented with 13.3 µM 6-benzylaminopurine and 4.5 µM thidiazuron.

Table 2.2 Effect of cytokinins on adventitious shoot regeneration from hypocotyls of white ash.

Plant Growth Regulator (μM)		Hypocotyl		
BA	TDZ	Shoot Formation[a,d] (%)	Shoot Elongation[b,d] (%)	Mean No. Shoots[c,d]
0.0	0.0	0.0 ± 0.0e	0.0 ± 0.0c	0.0 ± 0.0c
4.4	0.0	68.0 ± 6.8ab	32.7 ± 8.8ab	2.9 ± 1.0ab
8.9	0.0	61.3 ± 3.3abc	28.8 ± 11.0ab	2.1 ± 0.6abc
13.3	0.0	54.7 ± 2.5abcd	25.0 ± 8.6abc	2.2 ± 0.6abc
22.2	0.0	41.3 ± 7.1d	35.4 ± 9.6ab	4.6 ± 2.4a
0.0	0.5	73.3 ± 6.0a	19.4 ± 5.3bc	2.4 ± 0.7abc
4.4	0.5	54.7 ± 3.3abcd	19.7 ± 7.1bc	1.7 ± 0.6bc
8.9	0.5	62.7 ± 4.5abc	27.5 ± 10.6ab	3.2 ± 0.9ab
13.3	0.5	61.3 ± 5.7abc	32.7 ± 5.8ab	2.3 ± 0.5abc
22.2	0.5	52.0 ± 7.4bcd	46.9 ± 7.7a	3.9 ± 0.4ab
0.0	2.3	62.7 ± 5.4abc	18.6 ± 5.8bc	2.3 ± 0.8abc
4.4	2.3	54.7 ± 7.1abcd	16.3 ± 4.8bc	1.6 ± 0.9bc
8.9	2.3	62.7 ± 3.4abc	21.5 ± 5.6abc	2.5 ± 0.5abc
13.3	2.3	54.7 ± 4.4abcd	20.1 ± 11.9bc	2.1 ± 0.9abc
22.2	2.3	50.7 ± 10.7bcd	31.2 ± 6.2ab	1.4 ± 0.2bc
0.0	4.5	68.9 ± 4.6ab	23.8 ± 4.9abc	2.3 ± 0.7abc
4.4	4.5	52.0 ± 6.4bcd	42.6 ± 7.9ab	3.0 ± 0.6ab
8.9	4.5	52.0 ± 5.0bcd	22.1 ± 7.0abc	1.3 ± 0.3bc
13.3	4.5	68.0 ± 3.9ab	27.0 ± 5.3ab	1.5 ± 0.2bc
22.2	4.5	48.0 ± 5.7cd	41.6 ± 12.1ab	2.9 ± 0.7ab

[a]Mean ± standard error for 75 explants per treatment.
[b]Mean ± standard error for hypocotyls that produced leaf primordia and were able to elongate shoots.
[c]Mean number of shoots based on the number of hypocotyls transferred to Murashige and Skoog medium supplemented with 10 μM 6-benzylaminopurine (BA) and 10 μM thidiazuron (TDZ).
[d]Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$).

Table 2.3 Effect of auxin concentration on in vitro root formation of white ash microshoots.

Treatment					
Dark Period (days)	IAA + IBA (μM)	Rooting[a,b] (%)	Mean No. Roots[a,b]	Mean Root Length[a,b] (cm)	Mean No. Lateral Roots[a,b]
5	0.0 + 4.9	52.8 ± 10.6abc	1.8 ± 0.2b	4.0 ± 0.6ab	
5	2.9 + 4.9	80.6 ± 8.1a	2.5 ± 0.3ab	3.6 ± 0.7ab	9.1 ± 2.0a
5	5.7 + 4.9	77.8 ± 6.5a	2.6 ± 0.4ab	4.1 ± 0.8a	7.6 ± 1.5a
5	8.6 + 4.9	69.4 ± 10.0ab	3.6 ± 0.6a	2.7 ± 0.6abc	4.1 ± 1.1a
10	0.0 + 4.9	36.1 ± 12.6c	1.8 ± 0.5b	1.6 ± 0.7c	5.7 ± 3.2a
10	2.9 + 4.9	66.7 ± 9.3abc	2.3 ± 0.4ab	2.5 ± 0.6abc	5.8 ± 2.3a
10	5.7 + 4.9	63.9 ± 11.1abc	2.3 ± 0.4ab	2.1 ± 0.6bc	3.7 ± 1.6a
10	8.6 + 4.9	41.7 ± 11.8bc	1.4 ± 0.4b	1.2 ± 0.3c	4.1 ± 1.2a

[a]Values represent the means ± standard errors for 36 explants per treatment. IAA, indole-3-acetic acid; IBA, indole-3-butyric acid.
[b]Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$).

Figure 2.1 Callus formation and shoot initiation on a cotyledon explant of Fraxinus americana. Cotyledon cultured for 4 wk on Murashige and Skoog medium with 13.3 µM 6-benzylaminopurine plus 4.5 µM thidiazuron.

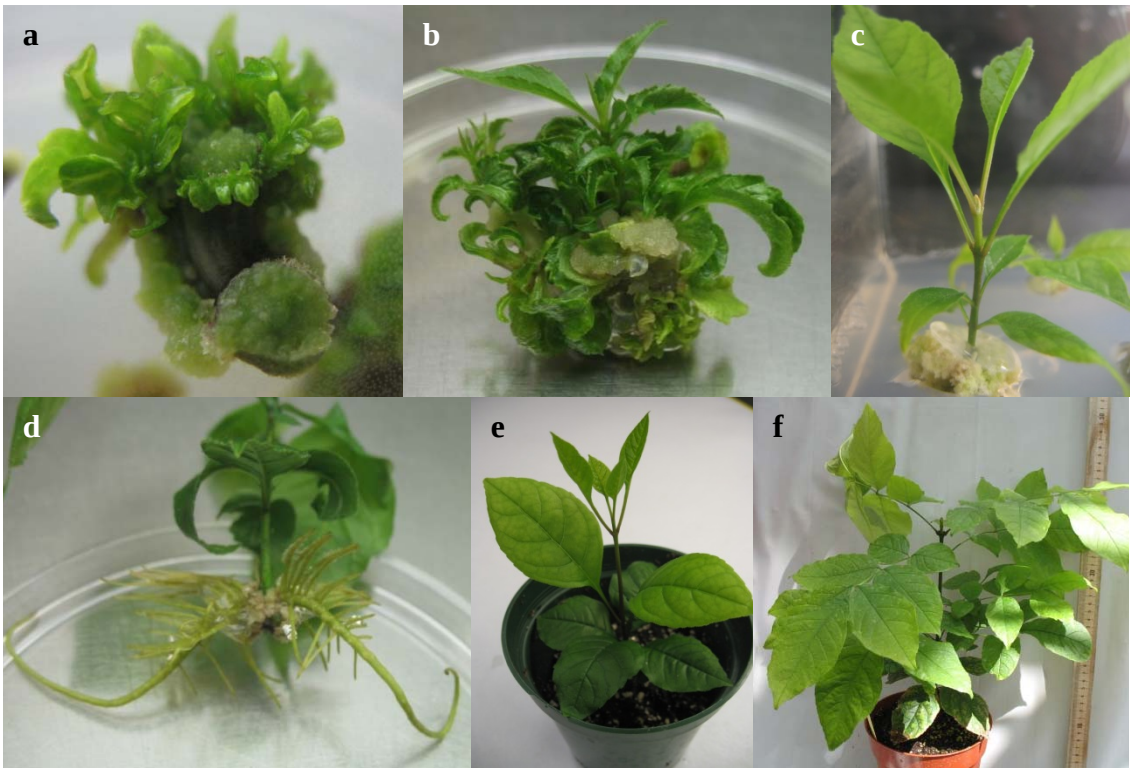

Figure 2.2 Plant regeneration from hypocotyls of Fraxinus americana. (a) Callus formation and adventitious shoot induction on a hypocotyl explant after 4 wk on Murashige and Skoog (MS) medium with 13.3 µM 6-benzylaminopurine (BA) plus 4.5 µM thidiazuron (TDZ). (b, c) elongation of adventitious shoots from hypocotyls on MS with 10 µM BA plus 10 µM TDZ, (d) shoot with roots on woody plant medium with 2.9 µM indole-3-acetic acid plus 4.9 µM indole-3-butyric acid after 5 d in the dark and then 4 wk under a 16-h photoperiod, (e) acclimatized white ash plantlet in the culture room producing simple leaves, (f) acclimatized white ash plant in the greenhouse exhibiting compound leaf growth.

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CHAPTER 3. *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *FRAXINUS AMERICANA* HYPOCOTYLS

3.1 Abstract

Fraxinus americana (white ash) trees provide both economic and ecological benefits. White ash is a valuable hardwood tree species that provides both food and shelter for wildlife, and the wood is highly valued in the hardwood industry. The emerald ash borer (EAB) is an invasive non-native beetle that threatens all *Fraxinus* species growing in North America, and there are no known means of complete eradication of this beetle or any innate resistance in the native tree populations. Therefore, the development of white ash with resistance to the EAB is an urgent goal. Hypocotyls isolated from mature embryos germinated on Murashige and Skoog (MS) medium supplemented with 22.2 μM 6-benzyladenine (BA) and 0.5 μM thidiazuron (TDZ) were transformed using *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pq35GR containing the neomycin phosphotransferase (*nptII*) and β-glucuronidase (GUS) fusion gene, as well as an enhanced green fluorescent protein (EGFP) gene. Explants were transformed in a bacterial suspension with 100 μM acetosyringone using 90 s sonication and 10 min vacuum-infiltration. Timentin at 500 mg L^{-1} was optimal for controlling excess bacterial growth and transformed cells were selected using 30 mg L^{-1} kanamycin. Seven independent lines of transformed shoots were selectively regenerated on MS

medium with 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} adenine sulfate, 10% coconut water, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin. The presence of GUS, *nptII*, and EGFP genes were confirmed by polymerase chain reaction (PCR). Reverse transcription – PCR analysis demonstrated the expression of the EGFP gene, and the protein was visualized in the cells of transgenic leaves. Transgenic microshoots were rooted (80.0%) on woody plant medium with 4.9 µM indole-3-butyric acid, 2.9 µM indole-3-acetic acid, and 500 mg L^{-1} timentin and subsequently acclimatized to the culture room. This protocol provides the framework for genetic transformation of white ash with a gene specific for EAB resistance.

3.2 Introduction

White ash (*Fraxinus americana* L.) is the most common and economically valuable of the 16 ash species native to North America. The wood is valued for the production of tool handles, furniture, flooring, boats, and baseball bats. Considered one of the best soil-improving species because of its calcium-rich leaves and earthworm palatability, the tree is also utilized as shelter and sustenance by a variety of wildlife. The vibrant fall color, relatively fast growth, and tolerance to a wide range of soil pH make it a favorite of the horticulture industry (Johnston 1939; Schlesinger 1990; Nesom 2001; Wallander 2008). In 2005, ash trees composed 5% to 29% of all street trees in the Midwest, replacing many of the elm trees in urban settings since the advent of Dutch elm disease (MacFarlane and Meyer 2005).

North American ash resources are currently being threatened with extinction by the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). An

aggressive, wood-boring exotic, the beetle has spread rapidly throughout at least 18 states (Connecticut, Illinois, Indiana, Iowa, Kansas, Kentucky, Maryland, Massachusetts, Michigan, Minnesota, Missouri, New York, Ohio, Pennsylvania, Tennessee, Virginia, West Virginia, and Wisconsin) and parts of Canada (Ontario and Quebec) since being discovered in southeastern Michigan in 2002 (Yu 1992; Emerald Ash Borer Information Website 2013). Adult beetles feed on the tree's foliage and bore holes into the bark, but the most damage is done by the larvae that feed on the phloem tissue, forming galleries and disrupting the flow of nutrients between shoot and root (McCullough and Katovich 2004). EAB is fatal to ash trees, and there is neither any known innate resistance in native trees nor any means to completely eradicate the beetle. Over 99% tree mortality in a stand was probable within 4 years of the first observed symptoms of infestation (Knight et al. 2012).

There has been little impact made on EAB populations or infestation rates from biological control and eradication efforts, and the beetle's range is rapidly expanding (Dobesberger 2002; Poland and McCullough 2006; Duan et al. 2012). Chemical controls were deemed expensive and inappropriate for protecting ash trees in forested or riparian areas, as well as environmentally undesirable (Poland and McCullough 2006; Bauer and Londoño 2011; Hahn et al. 2011). The predicted cost of treating, removing, and replacing urban forest trees affected by EAB was expected to exceed $10 billion by 2019 (Kovacs et al. 2010). Genetically engineering ash trees to express resistance genes such as the *Bacillus thuringiensis Cry* genes is a desirable alternative for obtaining a cost-efficient, effective, and environmentally friendly means of controlling EAB damage in the North American landscape.

To date, genetic transformation has been attempted for several *Fraxinus* spp., however transgenic shoots have only been reported for *F. pennsylvanica* and *F. profunda* (Roome 1992; Bates 1997; Du and Pijut 2009; Stevens 2012). Bates (1997) obtained multiple shoots after transforming white ash with *Agrobacterium*, but no shoots were confirmed transgenic. Our study is the first report of successfully regenerating transgenic white ash trees, and provides a foundation for integrating EAB resistance genes into these trees.

3.3 Materials and Methods

3.3.1 Plant Materials

Mature white ash seeds purchased in 2011 from Sheffield's Seed Co., Inc. (Locke, NY) were stored in a sealed container in the dark at 5°C until used. Disinfestation, isolation of embryos, and adventitious shoot regeneration were achieved using our previously described protocols (Palla and Pijut 2011). Freshly isolated aseptic embryos were cultured vertically in Magenta GA-7 vessels (Magenta Corp., Chicago, IL; 25 embryos per vessel) containing 50 mL (pre-culture) Murashige and Skoog (1962) (MS) medium (M499; PhytoTechnology Laboratories, Shawnee Mission, KS) supplemented with MS organics, 22.2 µM 6-benzyladenine (BA), and 0.5 µM thidiazuron (TDZ) for 5- to 15-d. Hypocotyls of in vitro germinated seedlings were then isolated and cultured horizontally on medium in order to determine explant sensitivity to kanamycin and timentin for transformation and selection procedures. All plant materials were cultured on medium containing 30 g L^{-1} sucrose and 7 g L^{-1} Bacto agar (No. 214030; Becton Dickinson,

USA), with the pH adjusted to 5.7 prior to autoclaving, unless otherwise noted, and all plant cultures were grown at 24 ± 2°C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}).

3.3.2 Effect of kanamycin and timentin on hypocotyl regeneration

Hypocotyl explants from 5-d-old in vitro grown seedlings were cultured horizontally on MS medium supplemented with 22.2 μM BA, 0.5 μM TDZ, 50 mg L^{-1} adenine hemisulfate (AS), 10% (v/v) coconut water (CW) (C195; PhytoTechnology Laboratories, Shawnee Mission, KS) with kanamycin (0, 5, 10, 15, 20, 30, 40, or 50 mg L^{-1}) or timentin (0, 100, 200, 300, 400, 500, or 600 mg L^{-1}) in Petri plates (100 x 25 mm; 50 mL medium). The antibiotics were each dissolved in sterile, deionized water and filter-sterilized (0.22 μM) before being added to autoclaved medium. Three replicates with 12 hypocotyls each were cultured for each treatment to determine the optimum concentration of antibiotics to be used in transformation and selection procedures. Regeneration response (percent callus and shoot formation) was evaluated and recorded after 6 wk culture in vitro.

3.3.3 Transformation vector and culture

The pq35GR vector (Fig. 3.1) was comprised of the cauliflower mosaic virus (CaMV) 35S promoter-derived bi-directional promoters containing two divergently arranged enhancer repeats, as well as an enhanced green fluorescent protein (EGFP) gene, and a fusion between the neomycin phosphotransferase (*nptII*) and *β*-glucuronidase (GUS) genes. Introduced into *Agrobacterium tumefaciens* strain EHA105, the plasmid was used for plant transformation (Li et al. 2004; Du and Pijut 2009). Single *Agrobacterium* colonies were cultured in the dark for 2 d on a rotary shaker (150 rpm) at 28°C in 20 mL YEP medium (10 g L^{-1} yeast extract, 10 g L^{-1} bacto-peptone, 5 g L^{-1} NaCl, pH 7.0) plus

20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin. The *Agrobacterium*-pq35GR suspension (OD_{600} = 0.4 – 0.6) was centrifuged at 3,000 rpm for 15 min, and the pellet was re-suspended in 20 mL liquid MS co-culture medium containing 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} AS, 10% CW, with the addition of 100 µM acetosyringone. The bacterial suspension was agitated for 1 h on the rotary shaker (150 rpm) prior to co-cultivation with hypocotyls.

3.3.4 Transformation and transgenic shoot regeneration

Hypocotyls excised from 5- to 15-d-old germinated embryos were placed in 20 mL liquid medium [MS medium with 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} AS, and 10% CW] and sonicated for 90 s before being transferred to the liquid *Agrobacterium*-pq35GR suspension and vacuum-infiltrated (62.5 cm Hg) for 10 min. Explants were then blotted dry on sterile filter paper and cultured on semi-solid MS co-culture medium in Petri plates (100 x 15 mm; 30 mL; 50 explants per plate) and cultured in the dark for 2-3 d at 27°C. Hypocotyls were then washed three times with liquid co-culture medium to remove excess bacterial growth, blotted dry, and cultured horizontally on selection medium [MS medium supplemented with 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} AS, 10% CW, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin; 15-20 explants per plate] for up to 8 wk. Explants regenerating shoot primordia were moved to selection elongation medium [MS medium with Gamborg et al. (1968) B5 vitamins plus 0.002 g L^{-1} glycine (MSB5G) supplemented with 10 µM BA, 10 µM TDZ, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin]. Elongating, kanamycin-resistant shoots were evaluated for the presence of marker genes after six or more subcultures (4 wk interval) on selection elongation medium. Shoots confirmed to contain transgenes were moved to multiplication medium

[MSB5G medium supplemented with 10 µM BA, 10 µM TDZ, and 500 mg L^{-1} timentin] to increase proliferation rates.

3.3.5 Effect of cytokinin concentration on shoot elongation

To maximize the number of microshoots elongating from transformed hypocotyls, a factorial experiment was performed to determine the optimal concentrations of BA and TDZ. Explants initiating shoot buds on selection medium (4-8 wk) were transferred to MSB5G medium supplemented with 30 mg L^{-1} kanamycin, 500 mg L^{-1} timentin, BA (0, 10, 15, or 20 µM) in combination with TDZ (0, 2.5, 5, or 10 µM) to induce shoot elongation. Magenta GA-7 vessels containing 50 ml of medium were used, with one explant per vessel. Eight explants per treatment were tested. Cultures were incubated for 6 wk and the number of shoots elongated was recorded.

3.3.6 Rooting of transgenic microshoots

Elongated transgenic shoots (3-5 cm; with at least two nodes) were pooled and randomly cultured on woody plant medium (WPM; L154; PhytoTechnology, Shawnee Mission, KS) (Lloyd and McCown 1981) supplemented with 500 mg L^{-1} timentin, 4.9 µM indole-3-butyric acid (IBA) plus 0, 2.9, 5.7, or 8.6 µM indole-3-acetic acid (IAA). Magenta GA-7 vessels containing 50 mL of medium were used, with one explant per vessel. Cultures were placed in the dark at 26°C for 5 d, then cultured up to 12 wk in the light. An overlay of 5 mL liquid rooting medium was added to cultures each week after 6 wk in light culture. Rooting percentage, the number of roots per shoot, and root length were recorded once roots were determined comparable to previous experiments (Palla and Pijut 2011). Three replications with five shoots per treatment per replication were

conducted. Rooted microshoots were acclimatized to ambient culture room conditions as described previously (Palla and Pijut 2011).

3.3.7 Histochemical GUS assay

Transformed plant tissue (callus, hypocotyl, and leaf primordia) regenerating on selection medium containing kanamycin was incubated overnight at 37°C in a staining solution (0.1 M $NaHPO_4$ buffer (pH 7.0), 0.5 mM $K_3[Fe(CN)_6]$, 0.5mM $K_4[Fe(CN)_6]$, 10 mM EDTA, 800 mg L^{-1} X-Gluc, 0.06% (v/v) Triton X-100) following the procedures described by Jefferson et al. (1987). Chlorophyll was removed from the tissue after staining using 20% (v/v) commercial bleach solution (5.25% sodium hypochlorite) for 15-20 min to allow for visualization and documentation of GUS expression.

3.3.8 EGFP fluorescence and imaging

The presence of GFP was detected using a fluorescence stereomicroscope (Leica MZFLIII) equipped with a 470/40 nm excitation filter (GFP-Plant) and a 525/50 nm barrier filter, and lit with an HBO 100 W mercury bulb. Leaves from transgenic plants confirmed to contain the *nptII*, GUS, and EGFP genes were examined alongside control non-transgenic leaves, and the presence or absence of green fluorescence was compared. No interference filter was used to block chlorophyll autofluorescence.

3.3.9 Molecular analysis of transgenic plant lines

Genomic DNA was isolated from leaves of seven independent putative transgenic lines and from one control, non-transformed plant following either the protocol of the Qiagen DNeasy Plant Mini Kit (Qiagen, USA) or the procedures described by Lefort and Douglas (1999). A polymerase chain reaction (PCR) was performed to amplify DNA sequences specific to the *nptII*, GUS, and EGFP genes. Primers (forward primer *nptII*-F

5’-TGCTCCTGCCGAGAAAGTAT-3’ and reverse primer *nptII*-R 5’-AATATCACGGGTAGCCAAGC-3’) were designed to amplify a 364 bp PCR product for the *nptII* gene. Primers (forward primer GUS-F 5’-TGCTGTCGGCTTTAACCTCT-3’ and reverse primer GUS-R 5’-GGCACAGCACATCAAAGAGA-3’) were designed to amplify a 332 bp PCR product for the GUS gene. Primers (forward primer EGFP-51 5’-ATGGTGAGCAAGGGCGAGGAGCTGT-‘3 and reverse primer EGFP-32 5’-TTACTTGTACAGCTCGTCCATGCCG-‘3) were designed to amplify a 720 bp PCR product for the EGFP gene. The PCR reaction (25 µL) consisted of 2.5 µL 10x PCR buffer (Invitrogen), 1 µL 50 mM Mg^{2+}, 1 µL 50 mM dNTP, 1 µL each of 10 µM *nptII*-F and *nptII*-R or 10 µM GUS-F and GUS-R or 10 µM EGFP-51 and EGFP-32 primers, 2 µL DNA template (100 ng μL^{-1}), 0.25 µL 5 U μL^{-1} Taq polymerase, and 16.25 µL sterile-deionized water. Plasmid DNA was used as a positive control; DNA from a non-transformed plant and sterile-deionized water served as negative controls. The *nptII* PCR reaction included 2 min at 94°C, followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 2 min, with a final 10 min cycle at 72°C. The GUS PCR reaction varied in the number of cycles run (45) and the annealing temperature used (55°C), but was otherwise identical. The EGFP PCR reaction included 4 min at 95°C, followed by 40 cycles of 95°C for 30 s, 64°C for 30 s, 72°C for 1 min, with a final 7 min cycle at 72°C. Amplified products of the PCR were visualized under UV light after gel electrophoresis (1% w/v agarose plus 0.5 µg mL^{-1} ethidium bromide).

Reverse transcription – PCR (RT-PCR) was performed on transgenic plant lines to confirm transgene expression. RNA was isolated from leaves (100 mg) of microshoots confirmed by PCR to contain transgenes, and one control, non-transformed plant

following the protocol of the Qiagen RNeasy Plant Mini Kit (Qiagen, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA) to remove genomic DNA. DNase-treated RNA (5 μg) was used as a template for reverse transcription to synthesize complementary DNA (cDNA) with AccuScript High Fidelity 1st Strand cDNA Synthesis kit (Agilent Technologies, USA) according to the manufacturer's instructions. PCR was performed using cDNA created with Oligo (dT) primers to amplify sequences specific to the EGFP gene. PCR was also performed to amplify sequences specific to a housekeeping 26S ribosomal RNA (rRNA) gene using cDNA created with random primers. PCR to amplify the 720 bp product for the EGFP gene was carried out using the respective primers and thermal cycle as described previously. Primers (forward primer 26S-F 5'-GTCCTAAGATGAGCTCAA-'3 and reverse primer 26S-R 5'-GGTAACTTTTCTGACACCTC-'3) were designed to amplify a 160 bp PCR product for the 26S rRNA gene. The PCR reaction (25 μL) consisted of 2.5 μL 10x PCR buffer (Invitrogen), 1 μL 50 mM Mg^{2+}, 1 μL 50 mM dNTP, 1 μL each of 10 μM 26S-F and 26S-R or 10 μM EGFP-51 and EGFP-32 primers, 3 μL cDNA template (5 ng μL^{-1}), 0.25 μL 5 U μL^{-1} Taq polymerase, and 15.25 μL sterile-deionized water. Plasmid DNA was used as a positive control; cDNA from a non-transformed plant and sterile-deionized water served as negative controls; DNase-treated RNA that had not been reverse transcribed served as template control to monitor DNA contamination. The 26S PCR reaction included 4 min at 95°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, with a final 7 min cycle at 72°C. Amplified products of the PCR were visualized under UV light after gel electrophoresis (1% agarose (w/v) plus 0.5 μg mL^{-1} ethidium bromide).

3.3.10 Statistical analysis

SPSS (Software Version 20) (SPSS 2011) was used to analyze data. An analysis of variance (ANOVA) was performed using the General Linear Model procedure on the individual replicate means by treatment for percent shoot formation, number of shoots per hypocotyl, percent root formation, number of roots per shoot, root length, and number of lateral roots. When the ANOVA indicated statistical significance, a Duncan's comparison test with an alpha level of 0.05 was used to distinguish the differences between treatments.

3.4 Results and Discussion

3.4.1 Effect of kanamycin and timentin on ash hypocotyl regeneration

Hypocotyls were exposed to various concentrations of kanamycin and timentin in order to determine the effect that the antibiotics had on regeneration of white ash adventitious shoots. Kanamycin is commonly used in the selection of transformed cells, inhibiting the growth of cells that do not have the *nptII* gene integrated, and concentration is critical for this selection. White ash callus and shoot regeneration were significantly inhibited when kanamycin concentrations reached 30 mg L^{-1} (Table 3.1). No shoot primordia were able to regenerate at that concentration and callus production was severely limited, with the majority of hypocotyls turning chlorotic and necrotic after 6 wk of culture. Thus, we determined 30 mg L^{-1} kanamycin to be the optimal concentration to use for subsequent transformation and selection, as it provided a high selection pressure to ensure regenerating shoots would be stable transformants instead of potential escapes. This

agrees with the previous study on white ash, where Bates (1997) noted organogenic inhibition in seedlings cultured on kanamycin concentrations ≥ 20 mg L^{-1}. Other ash studies reported kanamycin inhibited organogenesis at concentrations of 20 mg L^{-1} (Du and Pijut 2009; Stevens 2012). Roome (1992) observed that kanamycin as low as 10 mg L^{-1} limited green ash shoot production, and was unable to regenerate any shoots at higher concentrations.

Timentin was used in this study to inhibit growth of *Agrobacterium* after transformation. White ash hypocotyls were not significantly inhibited by timentin, and the highest shoot primordia regeneration (80.6 %) occurred on medium containing 500 mg L^{-1} (Table 3.2). Similar high concentrations (300 - 400 mg L^{-1}) were noted to have little effect on hypocotyl regeneration for other ash and hardwood species (Gonzalez Padilla et al. 2003; Andrade et al. 2009; Du and Pijut 2009; Stevens 2012).

3.4.2 Transformation and regeneration of plants

Germination of mature white ash seedlings was previously noted to be variable, with a delay of 11- to 21-d before being sorted to use for transformation (Bates 1997). By culturing aseptically extracted embryos on pre-culture medium, not only were embryos ready for transformation in 5 d, but germination was almost entirely uniform. Embryos could be grown on this medium for up to 15 d to allow for further hypocotyl growth. This extended pre-culture may help the tissue better survive the transformation process (data not shown). Green ash hypocotyls were seen to be hypersensitive to *Agrobacterium*, but a brief pre-culture period prior to inoculation attenuated the response (Du and Pijut 2009). The medium used to germinate the embryos in this study appeared to serve the same purpose as a pre-culture period, allowing hypocotyls to be freshly isolated and used for

transformation. Combining embryo germination with a pre-culture step was shown to be beneficial for plum (Gonzalez Padilla et al. 2003) and pumpkin ash (Stevens 2012).

Since *Agrobacterium* was shown to be attracted to the compounds released by wounded plants (Satchel et al. 1985), the use of freshly isolated hypocotyls increased the number of sites where inoculation can potentially occur. Roome (1992) used abrasive celites to wound explants in one transformation study, while freshly cut hypocotyls proved effective for Gonzalez Padilla et al. (2003). Sonication was used in this study to further increase the number of superficial wound sites where gene transfer could take place, and was combined with vacuum-infiltration to force the *Agrobacterium* cells into further contact with the white ash hypocotyl cells. Sonication in conjunction with vacuum-infiltration has proven valuable for the transformation of many species, particularly when thicker explant tissue such as hypocotyls have been used (Andrade et al. 2009; Du and Pijut 2009; Subramanyam et al. 2011; Stevens 2012).

White ash hypocotyls were co-cultivated for 2- to 3-d after being exposed to an *Agrobacterium* solution with an $OD_{600} = 0.4 – 0.6$ to allow for gene transfer and integration. Higher *Agrobacterium* concentrations and longer co-culture periods resulted in an excess of bacterial growth during the selection process, regardless of timentin concentration, resulting in necrosis of explant tissue (data not shown). Similar bacterial concentrations (Sun et al. 2011) and co-culture times (Du and Pijut 2009; Stevens 2012) have been used with success.

Six weeks after culture on selection medium, 105 explants out of the 537 not lost to contamination were producing callus or shoot primordia and were moved to selection and elongation medium. Seven independent transgenic lines were recovered after 8 wk (1.3%

transformation efficiency). Adventitious shoots that elongated in the presence of kanamycin were continuously cultured every 3 or 4 wk on selection elongation medium for six or more subcultures. Shoot elongation occurred at a slow rate while on medium containing kanamycin, particularly for three out of the seven independent lines, although shoots were green and normal in appearance (Fig 3.2). The optimal concentration of BA and TDZ for shoot elongation was examined and determined to be 10 µM each (37.5%; 1.3 ± 0.6 shoots per explant; Table 3.3). Liquid overlays of elongation medium have been shown to increase the growth rate of transformed shoots (Stevens 2012), but failed to promote white ash shoot growth, instead causing excess callus formation at the base of microshoots. Once microshoots had grown large enough to remove leaf material without damaging growth, PCR was performed to confirm the presence of transgenes, and then all transgenic shoots were cultured on elongation medium without kanamycin. Shoot elongation recovered to a normal rate after 2- to 3-subcultures and were propagated in vitro for use in rooting trials (Palla and Pijut 2011).

Optimal rooting (80%) of the seven transgenic white ash lines recovered in this study occurred on WPM supplemented with 500 mg L^{-1} timentin, 4.9 µM IBA, and 2.9 µM IAA (Table 3.4). These results follow what was observed in previous regeneration work (Palla and Pijut 2011). Kanamycin has been known to negatively affect rooting (Gonzalez Padilla et al. 2003), and was observed to limit shoot callusing, root initiation, and root elongation in initial rooting tests with white ash transformants obtained in our lab (data not shown). All shoots used in the current rooting trial were derived from lines PCR-confirmed to contain transgenes, therefore kanamycin was omitted from the rooting medium to enhance root initiation and elongation. As with shoot elongation, root

formation and development was slow for the transformed shoots. Liquid overlays of medium were observed to enhance rooting in green ash (Kim et al. 1998). A liquid overlay of rooting medium (5 mL) every week after the initial 6 wk on rooting medium proved beneficial for elongating transgenic white ash roots, as well as maintaining the health of the shoot during culture. All roots that formed were firmly attached (Fig. 3.2c), although callus formation was more abundant at the base of the transgenic shoot and roots were thicker than had been observed during previous regeneration work (Palla and Pijut 2011). Some shoots exhibited root initiation from nodal or internodal stem portions in contact with the rooting medium, rather than from the basal end of the shoot. Rooted plantlets were acclimatized (100%) to ambient culture room conditions (Fig. 3.2d, e).

3.4.3 Analysis of transgenic plant lines

Transformed plant tissue on selection medium containing kanamycin was exposed to a histochemical GUS assay 4- to 6-wk after initial culture. Transient GUS expression was observed as a bright blue coloration in the tissue after chlorophyll removal. Staining was apparent within hypocotyl tissue, showing the successful transformation with *Agrobacterium* into the plant tissue, as well as on regenerating callus and leaf primordia, visualizing the transient expression of the GUS gene (Fig. 3.3).

Genomic DNA extracted from the leaves of seven putative transgenic lines was used in PCR analysis along with a non-transformed white ash plant. PCR amplification resulted in 332 bp (Fig. 3.4 lanes 4-10), 364 bp (Fig. 3.5 lanes 4-10), and 720 bp (Fig. 3.6 lanes 4-10) DNA fragments from all seven independent lines, corresponding to the GUS, *nptII*, and EGFP gene targets, respectively. PCR products of the corresponding size were found in the plasmid control (Fig. 3.4; 3.5; 3.6 lane 1), but absent in both the non-transformed

plant (Fig. 3.4; 3.5; 3.6 lane 3) and the water control (Fig. 3.4; 3.5; 3.6 lane 2). This confirmed the successful integration of the foreign genes of interest into *F. americana*.

Lines confirmed to be transgenic were further analyzed using a stereomicroscope with a 470/40 nm excitation filter to visualize fluorescence associated with the presence of the EGFP gene. Young leaves from PCR-confirmed shoots were compared *in vivo* against non-transformed control leaves. Bright green portions of fluorescence were apparent only on leaves from transgenic shoots (Fig. 3.7), confirming both the presence and the expression of the EGFP gene.

The expression of the EGFP gene in leaves of transgenic plants was also determined by RT-PCR analysis. The amplification of the 720 bp fragment of EGFP was found in all seven transgenic plant lines (Fig. 3.8 lanes 5-11). A corresponding band was found in the plasmid control (Fig 3.8 lane 4), but not in the water control (Fig. 3.8 lane 1), the RNA control (Fig. 3.8 lane 2), or the non-transformed plant (Fig. 3.8 lane 3). This confirmed the expression of the EGFP gene in all seven transformed lines, as well as the success of the reverse transcription of RNA into cDNA and the removal of DNA contamination. Yang et al. (2006) noted that *Agrobacterium* cells can potentially survive selection, persisting around the cells in leaves, stems, and roots of transgenic plants for 24 months while still in the presence of kanamycin. The highly conserved region of the housekeeping 26S rRNA found in plants was used as a control to validate RT-PCR results, as was used to evaluate the stable transformation of *Cornus* (Liu et al. 2013). All seven transgenic lines (Fig. 3.9 lanes 5-11) as well as the non-transformed plant (Fig. 3.9 lane 4) showed amplification of the 26S rRNA fragment, confirming that the gene expression detected during RT-PCR was the result of an integrated transgene rather than

Agrobacterium contamination. Contamination of the PCR reaction was apparent however, resulting in faint banding for the water control (Fig. 3.9 lane 2) and plasmid control (Fig. 3.9 lane 1). Several attempts have been made to eliminate the source of this contamination and obtain a clearer image.

3.5 Conclusions

Transgenic white ash plants were successfully developed using an *Agrobacterium*-mediated transformation and regeneration protocol for hypocotyl explants. Hypocotyls germinated up to 15 d on pre-culture medium containing cytokinins were exposed to sonication and vacuum-infiltrated with *Agrobacterium* strain EHA105 harboring the pq35GR vector. Selection of transformed cells was optimal with 30 mg L^{-1} kanamycin and excess *Agrobacterium* growth was controlled with 500 mg L^{-1} timentin without limiting organogenic potential. The integration of marker genes was confirmed through PCR analysis, GUS staining, GFP visualization, and RT-PCR analysis. Optimal rooting of transgenic shoots (80.0%) occurred on WPM with 500 mg L^{-1} timentin, 4.9 µM IBA plus 2.9 µM IAA. This is the first report of successfully regenerating transgenic white ash plants. Similar methods have been reported successful for green ash and pumpkin ash (Du and Pijut 2009; Stevens 2012). This protocol provides a method for future development of EAB-resistant white ash trees. Studies are currently underway to integrate the *Bacillus thuringiensis* Cry8Da toxin protein into *F. americana*, with the goal of imparting resistance to the beetle.

Table 3.1 Effect of kanamycin concentration on percent callus formation and shoot regeneration of Fraxinus americana hypocotyls.

Kanamycin (mg L^{-1})	Callus Formation[a] (%)	Shoot Formation[a,b] (%)
0	100.0 ± 0.0a	83.3 ± 9.6a
5	66.7 ± 8.3b	50.0 ± 4.8b
10	47.2 ± 10.0bc	27.8 ± 7.3c
15	52.8 ± 12.1bc	25.0 ± 9.6c
20	41.7 ± 8.3c	11.1 ± 4.8cd
30	19.4 ± 4.8d	0.0 ± 0.0d
40	0.0 ± 0.0d	0.0 ± 0.0d
50	0.0 ± 0.0d	0.0 ± 0.0d

Hypocotyls were placed on Murashige and Skoog medium supplemented with 22.2 µM 6-benzyladenine, 0.5 µM thidiazuron, 50 mg L^{-1} adenine hemisulfate, and 10% coconut water plus different concentrations of kanamycin. Data taken after 6 wk culture.

[a]Mean ± standard error for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$).
[b]Mean ± standard error for hypocotyls that produced leaf primordia.

Table 3.2 Effect of timentin concentration on percent callus formation and shoot regeneration of Fraxinus americana hypocotyls.

Timentin (mg L^{-1})	Callus Formation[a] (%)	Shoot Formation[a,b] (%)
0	100.0 ± 0.0a	91.7 ± 4.8a
50	100.0 ± 0.0a	50.0 ± 12.7c
100	100.0 ± 0.0a	72.2 ± 7.4abc
200	100.0 ± 0.0a	75.0 ± 4.8abc
300	100.0 ± 0.0a	75.0 ± 4.8abc
400	100.0 ± 0.0a	61.1 ± 10.0bc
500	100.0 ± 0.0a	80.6 ± 10.0ab
600	100.0 ± 0.0a	71.5 ± 3.4abc

Hypocotyls were placed on Murashige and Skoog medium supplemented with 22.2 µM 6-benzyladenine, 0.5 µM thidiazuron, 50 mg L^{-1} adenine hemisulfate, and 10% coconut water plus different concentrations of timentin. Data taken after 6 wk culture.

[a]Mean ± standard error for 36 explants per treatment. Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$).
[b]Mean ± standard error for hypocotyls that produced leaf primordia.

Table 3.3 Effect of cytokinin concentration on elongation of transgenic Fraxinus americana microshoots.

BA (μM)	TDZ (μM)	Shoot Elongation[a,b] (%)	Mean No. Shoot[a,b]
0.0	0.0	0.0 ± 0.0b	0.0 ± 0.0b
10.0	0.0	0.0 ± 0.0b	0.0 ± 0.0b
15.0	0.0	12.5 ± 70.7ab	2.0 ± 0.0a
20.0	0.0	12.5 ± 35.4ab	1.0 ± 0.0ab
0.0	2.5	0.0 ± 0.0b	0.0 ± 0.0b
10.0	2.5	0.0 ± 0.0b	0.0 ± 0.0b
15.0	2.5	12.5 ± 35.4ab	1.0 ± 0.0ab
20.0	2.5	0.0 ± 0.0b	0.0 ± 0.0b
0.0	5.0	0.0 ± 0.0b	0.0 ± 0.0b
10.0	5.0	0.0 ± 0.0 b	0.0 ± 0.0b
15.0	5.0	12.5 ± 70.7ab	2.0 ± 0.0a
20.0	5.0	0.0 ± 0.0b	0.0 ± 0.0b
0.0	10.0	0.0 ± 0.0b	0.0 ± 0.0b
10.0	10.0	37.5 ± 75.6a	1.3 ± 0.6ab
15.0	10.0	0.0 ± 0.0b	0.0 ± 0.0b
20.0	10.0	12.5 ± 35.4ab	1.0 ± 0.0ab

Hypocotyls forming shoot primordia were placed on Murashige and Skoog medium with Gamborg et al. (1968) B5 vitamins plus 0.002 g L^{-1} glycine supplemented with 30 mg L^{-1} kanamycin, 500 mg L^{-1} timentin, and various concentrations of 6-benzyladenine (BA) and thidiazuron (TDZ). Data taken after 6 wk culture.

[a]Values represent the means ± standard errors for 8 explants per treatment.
[b]Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$).

Table 3.4 Effect of auxin concentration on in vitro root formation of transgenic Fraxinus americana microshoots.

Treatment IAA + IBA (μM)	Rooting[a,b] (%)	Mean No. Roots[a,b]	Mean Root Length[a,b] (cm)	Mean No. Lateral Roots[a,b]
0.0 + 4.9	13.3 ± 13.3b	0.5 ± 0.4b	0.8 ± 0.1b	0.2 ± 0.1b
2.9 + 4.9	80.0 ± 11.5a	3.8 ± 0.8a	1.2 ± 0.3a	0.8 ± 0.3a
5.7 + 4.9	66.7 ± 13.3a	4.1 ± 1.3a	1.0 ± 0.2a	0.6 ± 0.2ab
8.6 + 4.9	53.3 ± 6.7a	3.1 ± 1.1ab	0.7 ± 0.2ab	0.2 ± 0.1b

Microshoots were placed on woody plant medium supplemented with 500 mg L^{-1} timentin, 4.9 μM indole-3-butyric acid (IBA), and various concentrations of indole-3-acetic acid (IAA). An overlay of liquid medium was added every week after 6 wk light culture. Data taken after 5 d dark culture followed by 8 - 12 wk light culture.

[a]Values represent the means ± standard errors for 15 explants per treatment.
[b]Means in each column followed by the same letter were not significantly different according to Duncan's multiple comparison test ($\alpha = 0.05$).

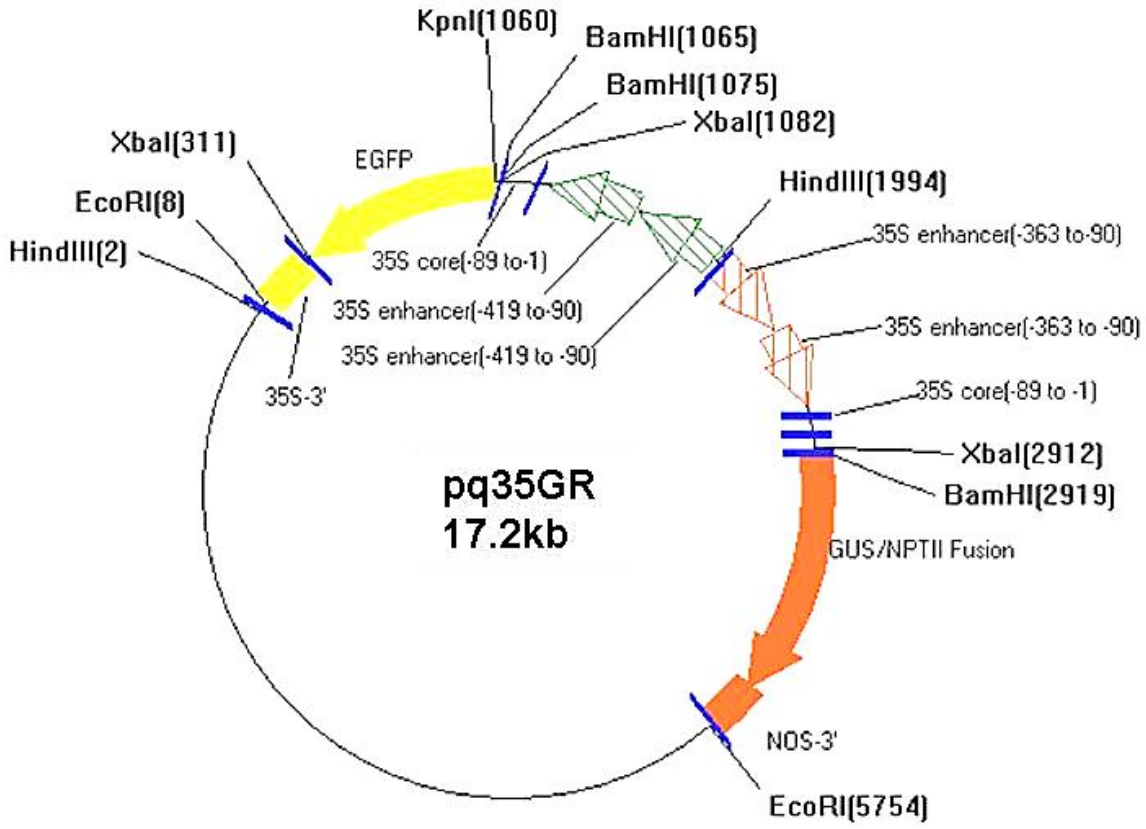

Figure 3.1 The pq35GR vector. The vector consisted of the cauliflower mosaic virus (*CaMV*) 35S promoter-derived bi-directional promoters containing two divergently arranged enhancer repeats, a fusion between the *nptII* and GUS genes, and the EGFP gene (Li et al. 2004).

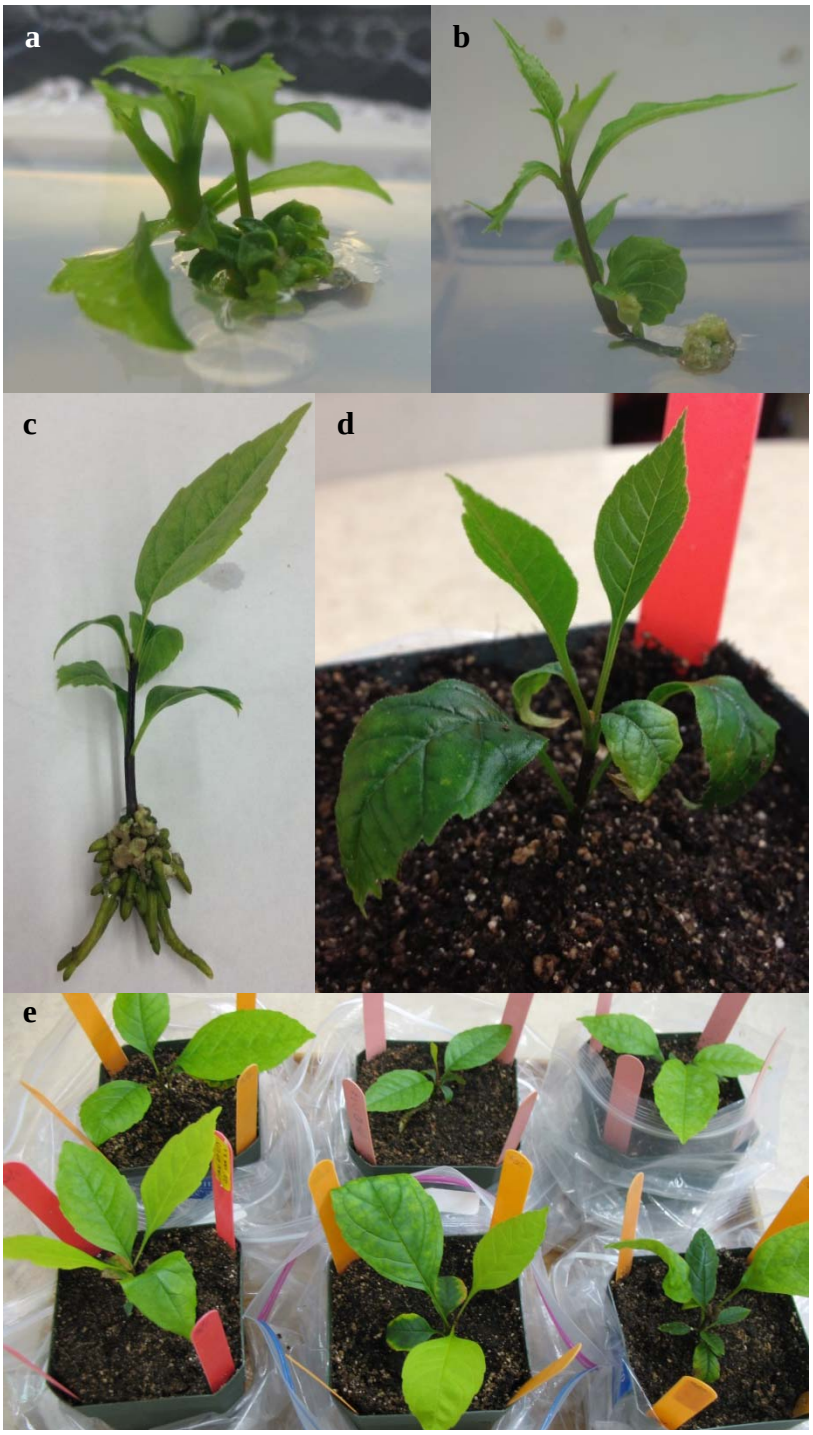

Figure 3.2 Kanamycin-resistant adventitious Fraxinus americana shoots. (a) Shoot elongating on selection elongation medium [MSB5G plus 10 µM BA, 10 µM TDZ, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin], (b) PCR-confirmed transgenic shoot proliferating on elongation medium without kanamycin, (c) root formation on PCR-confirmed transgenic shoot, (d, e) PCR-confirmed transgenic plants acclimatized to ambient culture room conditions.

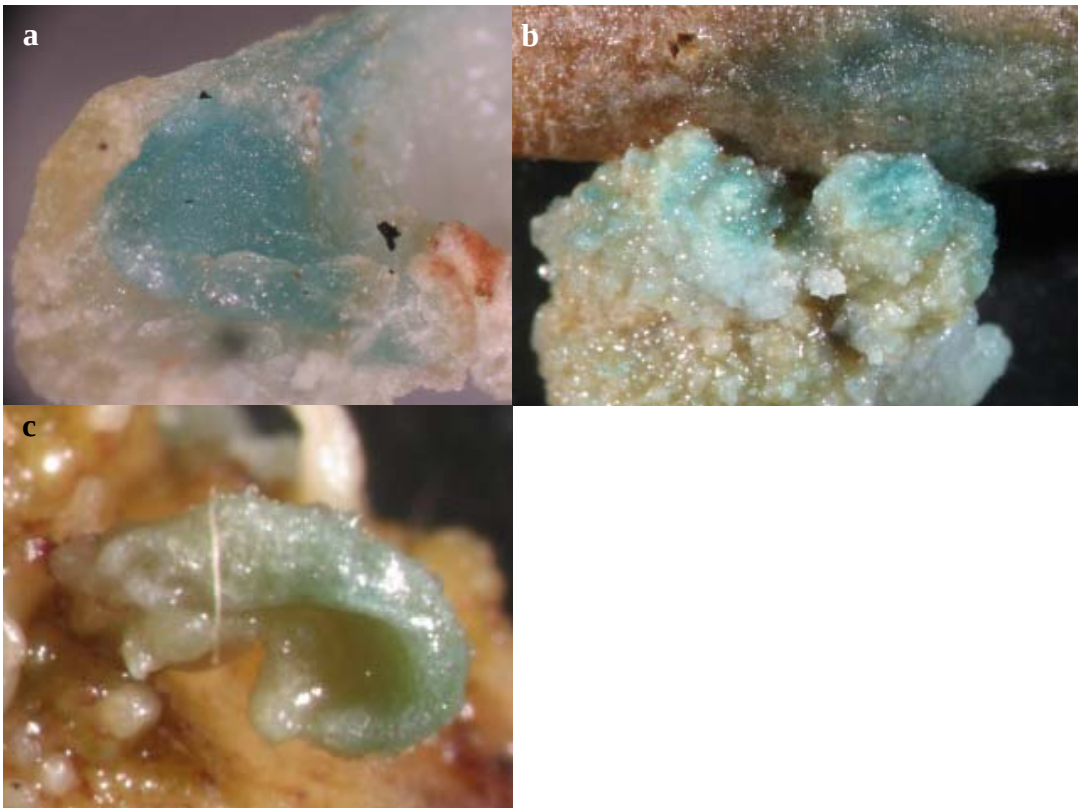

Figure 3.3 Transient GUS expression in Fraxinus americana. Expression in (a) hypocotyl tissue, (b) callus, and (c) leaf primordia.

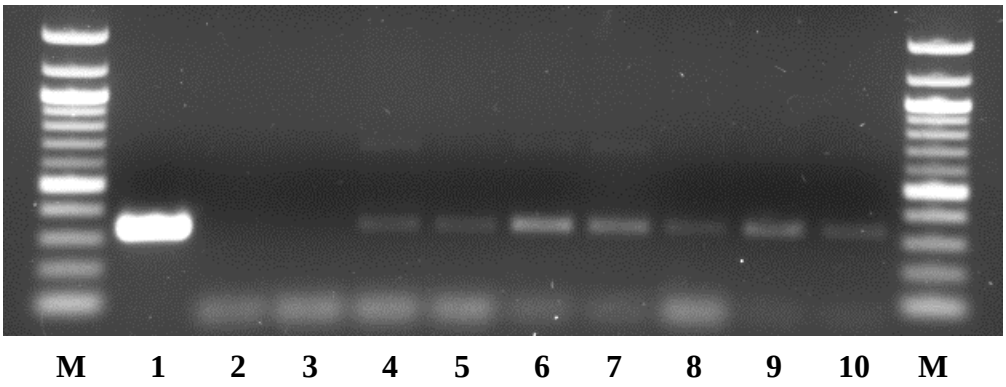

Fig 3.4 PCR analysis of genomic DNA isolated from leaves of non-transformed and transgenic white ash for amplification of 332-bp GUS gene. M 100 bp molecular marker, lane 1 positive control (pq35GR), lane 2 water control, lane 3 negative control (non-transformed plant), lanes 4-10 (putative transgenic shoots), M 100 bp molecular marker.

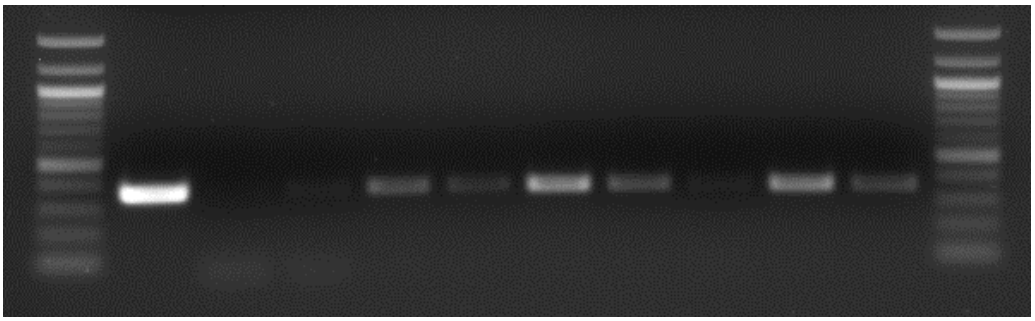

Fig 3.5 PCR analysis of genomic DNA isolated from leaves of non-transformed and transgenic white ash for amplification of 364-bp nptII gene. M 100 bp molecular marker, lane 1 positive control (pq35GR), lane 2 water control, lane 3 negative control (non-transformed plant), lanes 4-10 (putative transgenic shoots), M 100 bp molecular marker.

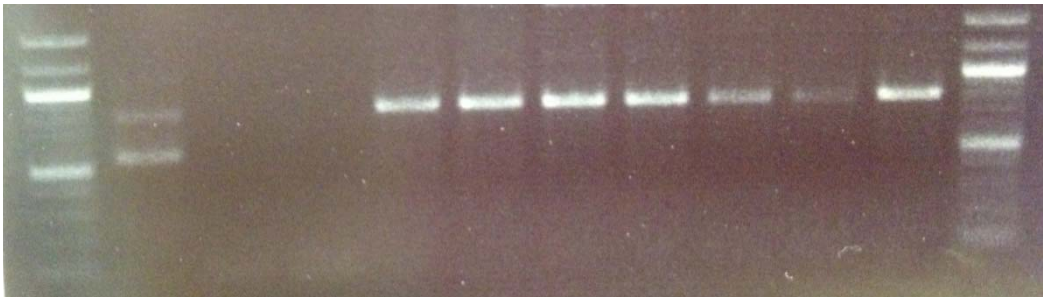

Fig 3.6 PCR analysis of genomic DNA isolated from leaves of non-transformed and transgenic white ash for amplification of 720-bp EGFP gene. M 100 bp molecular marker, lane 1 positive control (pq35GR), lane 2 water control, lane 3 negative control (non-transformed plant), lanes 4-10 (putative transgenic shoots), M 100 bp molecular marker.

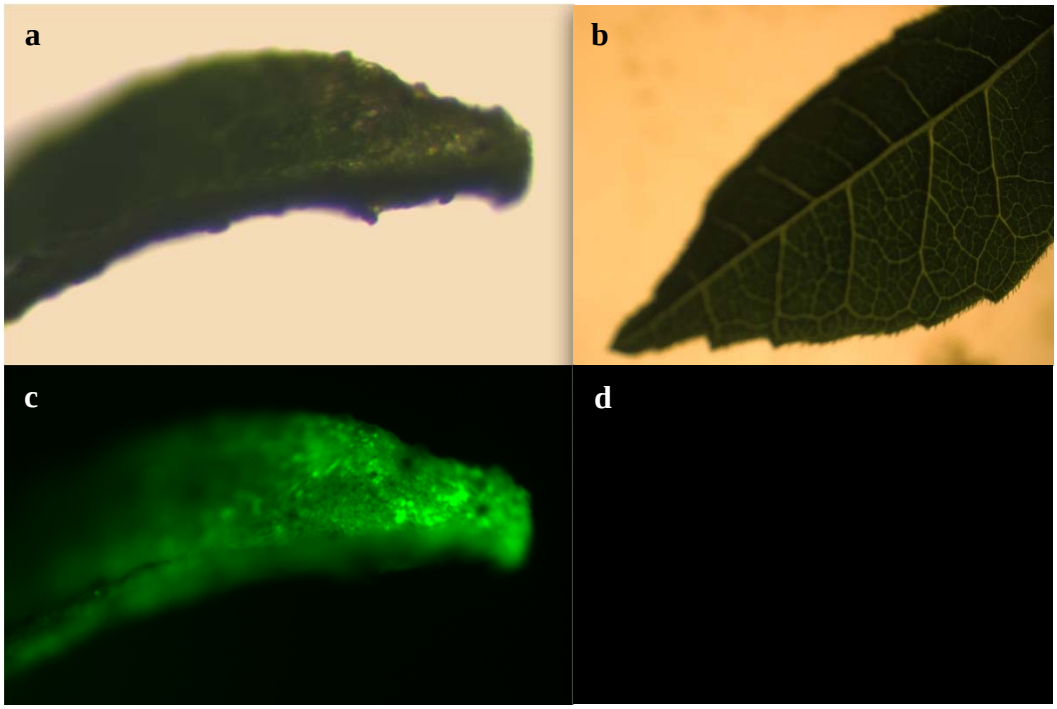

Figure 3.7 GFP visualization of transformed leaf. (a) Transformed leaf from microshoot confirmed to harbor GUS, *nptII*, and EGFP genes and (b) control non-transformed leaf under white light. Same (c) transformed leaf and (d) non-transformed leaf as seen through GFP excitation filter (470/40 nm).

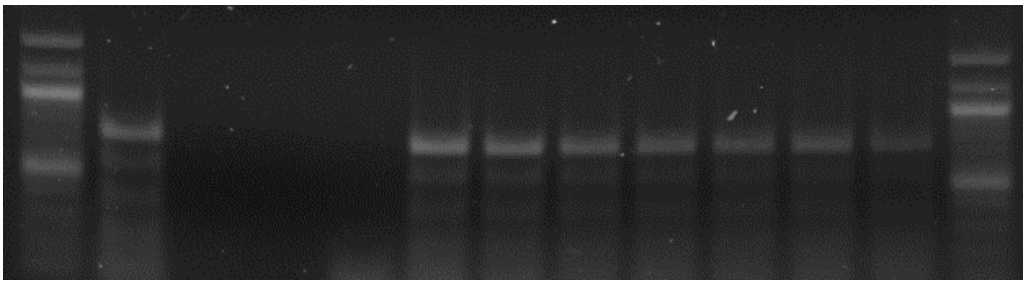

Fig 3.8 RT-PCR analysis of cDNA isolated from leaves of non-transformed and transgenic white ash for amplification of 720-bp EGFP gene. M 100 bp molecular marker, lane 1 water control, lane 2 DNase-treated RNA template control, lane 3 non-transformed plant (negative control), lane 4 pq35GR plasmid DNA (positive control), lanes 5-11 transgenic shoots, M 100 bp molecular marker.

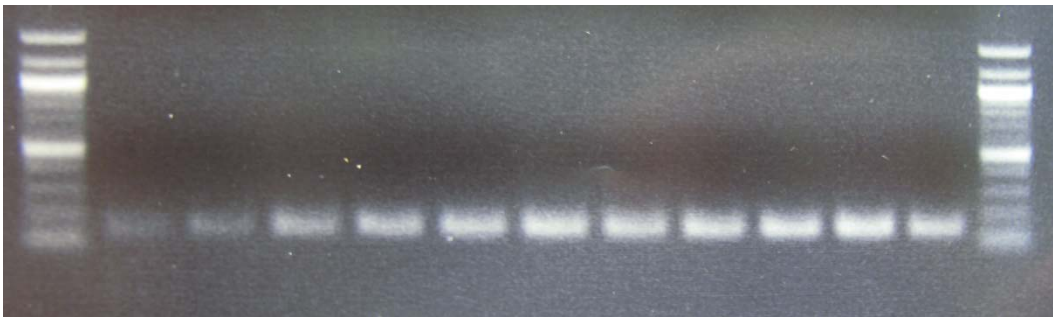

Fig 3.9 RT-PCR analysis of cDNA isolated from leaves of non-transformed and transgenic white ash for amplification of 160-bp 26S gene. M 100 bp molecular marker, lane 1 pq35GR plasmid DNA (negative control), lane 2 water control, lane 3 DNase-treated RNA template control, lane 4 non-transformed plant, lanes 5-11 transgenic shoots, M 100 bp molecular marker.

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CHAPTER 4. GENETIC TRANSFORMATION OF *FRAXINUS AMERICANA* FOR RESISTANCE TO THE EMERALD ASH BORER

4.1 Abstract

Hypocotyls isolated from mature embryos germinated on Murashige and Skoog (MS) medium supplemented with 22.2 μM 6-benzyladenine (BA) and 0.5 μM thidiazuron (TDZ) were transformed using *Agrobacterium tumefaciens* strain EHA105 harboring the pBI121-8D2 vector containing the neomycin phosphotransferase (*nptII*) gene and a full length, codon-optimized *Bacillus thuringiensis* Cry8Da protoxin gene (*cry8D2*). Explants were transformed in a bacterial suspension with 100 μM acetosyringone using 90 s sonication and 10 min vacuum-infiltration. Four independent lines of transformed shoots were selectively regenerated on MS medium with 22.2 μM BA, 0.5 μM TDZ, 50 mg L^{-1} adenine sulfate, 10% coconut water, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin. The presence of *cry8D2* and *nptII* were confirmed by polymerase chain reaction for all four transgenic lines. Transgenic white ash microshoots are being multiplied for use in future feeding trials to determine potential efficacy of the *cry8D2* gene against emerald ash borer attack.

4.2 Introduction

Fraxinus americana (white ash) trees provide both economic and ecological benefits. White ash provides food and shelter for wildlife, and is a hardwood tree species valued by both the hardwood industry and horticultural purveyors (Schlesinger 1990; Nesom 2001). The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive non-native beetle threatening all *Fraxinus* species growing in North America (Yu 1992). There are no known means of complete eradication of this beetle or of any innate resistance in the native tree populations, and current biological and chemical control programs have had little effect on EAB populations or infestation rates, allowing the beetle's range to expand rapidly (Dobesberger 2002; Poland and McCullough 2006; Bauer and Londoño 2011; Hahn et al. 2011; Duan et al. 2012).

Bacillus thuringiensis (*Bt*) is a naturally occurring soil bacterium that produces crystalline proteins (Cry toxins) during sporulation. Activated by specific proteases in the alkaline environment of an insect's gut, *Bt* proteins are toxic to specific insects, but harmless to mammals. These toxin proteins have potent insecticidal properties particular to a narrow taxonomic group of insects, with relatively good correlation to the host range at a family level (Schnepf et al. 1998; summarized in Sanahuja et al. 2011).

Genetic analysis of EAB populations in North America showed little diversity among the beetles (Bray et al. 2011). *Bt*-resistance in insect populations has only been observed after generations of intensive selection, and the use of preventative measures such as non-transgenic refuges to maintain non-resistant breeding individuals in the pest population have proven extremely effective in the agronomic crop industry (summarized in Sanahuja et al. 2011). Feral populations of ash could potentially benefit from mixed transgenic and

non-transgenic plantings, an effect that has been shown in commercial papaya, where trees transformed to be resistant to papaya ringspot virus kept infestation rates low enough to allow for non-resistant trees to be productive (Walter et al. 2010).

Insect resistance through the expression of *Bt* toxin proteins directly in plant tissue has been successful in crop plants (summarized in Sanahuja et al. 2011), as well as various species of *Picea*, *Pinus,* and *Populus* (Ellis et al. 1993; Kleiner et al. 1995; James et al. 1999; Génissel et al. 2003; Tang and Tian 2003; Grace et al. 2005; Lachance et al. 2007). The most effective resistance noted occurred when the *Bt* Cry gene used had been codon-optimized for use in plant systems and was under control of the cauliflower mosaic virus (CaMV) 35S promoter.

Laboratory bioassays have shown *Bt* SDS-502, a strain of *Bt* producing the Cry8Da toxin (Asano et al. 2003), to be virulent against EAB adults (Bauer and Londoño 2011). Genetically engineered ash trees expressing a Cry8Da protoxin gene are a desirable alternative for obtaining a cost-efficient, effective, and environmentally friendly means of controlling EAB damage in the North American landscape. Transgenic trees would be able to target the phloem-feeding larvae and would eliminate potential undesirable effects associated with external control methods.

Green ash (*F. pennsylvanica*) hypocotyls were transformed with the pBI121-8D2 vector, containing a codon-optimized, full length Cry8Da gene (*cry8D2*) (Du 2008). Three kanamycin-resistant green ash callus lines were confirmed to contain *cry8D2* through polymerase chain reaction (PCR), although no shoots were recovered. The present study is the first report of successfully regenerating transgenic white ash microshoots containing *cry8D2*, with potential resistance to EAB.

4.3 Materials and Methods

4.3.1 Plant Materials

Mature white ash seeds purchased in 2011 from Sheffield's Seed Co., Inc. (Locke, NY) were stored in a sealed container in the dark at 5°C until used. Disinfestation, isolation of embryos, and adventitious shoot regeneration were achieved using our previously described protocols (Palla and Pijut 2011). Freshly isolated aseptic embryos were cultured vertically in Magenta GA-7 vessels (Magenta Corp., Chicago, IL; 25 embryos per vessel) containing 50 mL (pre-culture) Murashige and Skoog (1962) (MS) medium (M499; PhytoTechnology Laboratories, Shawnee Mission, KS) supplemented with MS organics, 22.2 µM 6-benzyladenine (BA), and 0.5 µM thidiazuron (TDZ)] for 5- to 15-d prior to transformation. All plant materials were cultured on medium containing 30 g L^{-1} sucrose and 7 g L^{-1} Bacto agar (No. 214030; Becton Dickinson, USA), with the pH adjusted to 5.7 prior to autoclaving, unless otherwise noted, and all plant cultures were grown at 24 ± 2°C under a 16-h photoperiod (80 µmol m^{-2} s^{-1}).

4.3.2 Transformation vector construction and culture

The pBI121-8D2 vector (Fig. 4.1) was comprised of the CaMV 35S promoter, as well as the neomycin phosphotransferase (*nptII*) gene and a full length, codon-optimized Cry8Da protoxin gene (*cry8D2*). The pBI121 binary vector and pBI221-8D2 vector (Phyllom LLC, Mountain View, CA) were both digested with EcoRI (New England BioLabs, Ipswich, MA) for 1.5 h at 37°C. Samples were heat inactivated for 20 min at 65°C, then ethanol precipitated to remove enzyme and buffers from the DNA, and re-suspended in 30 µL sterile-deionized water. DNA fragments were then digested with

HindIII (New England BioLabs, Ipswich, MA) for 3 h at 37°C. Digestion products were run on a 1% (w/v) agarose gel and a 5KB band (pBI221-8D2) and a larger band (pBI121) were extracted with a QIAquick gel extraction kit (Qiagen, USA) before being ligated with T4 ligase overnight at 16°C. The ligation was transformed into competent DH5α *Escherichia coli* cells using the heat-shock method. Dilute and concentrated cells with successful ligation and transformation were cultured on Luria-Bertani (LB) medium (10 g L^{-1} bacto-tryptone, 5 g L^{-1} yeast extract, 10 g NaCl, pH 7.0) containing 20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin ($LBKan_{50}$) and incubated at 37°C overnight. Cultures of colonies in $LBKan_{50}$ were made and incubated overnight at 37°C (150 rpm). Colony-PCR was conducted for 8D2 and for *nptII* inserts confirmation. Plasmid DNA was isolated using a plasmid miniprep kit (Qiagen, USA) and introduced into *Agrobacterium tumefaciens* strain EHA105 using the heat-shock method. This pBI121-8D2 vector was then used for plant transformation. Single *Agrobacterium* colonies were cultured in the dark for 2 d on a rotary shaker (150 rpm) at 28°C in 20 mL Yeast Extract Peptone (YEP) medium (10 g L^{-1} yeast extract, 10 g L^{-1} bacto-peptone, 5 g L^{-1} NaCl, pH 7.0) plus 20 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin. The *Agrobacterium*-pBI121-8D2 suspension (OD_{600} = 0.4 – 0.6) was centrifuged at 3,000 rpm for 15 min, and the pellet was re-suspended in 20 mL liquid MS co-culture medium containing 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} adenine sulfate (AS), 10% coconut water (CW), with the addition of 100 µM acetosyringone. The bacterial suspension was agitated for 1 h on a rotary shaker (150 rpm) prior to co-cultivation with hypocotyls.

4.3.3 Transformation and transgenic shoot regeneration

Hypocotyls excised from 5- to 15-d-old germinated embryos were placed in 20 mL liquid medium [MS medium with 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} AS, and 10% CW] and sonicated for 90 s before being transferred to the liquid *Agrobacterium*-pBI121-8D2 suspension and vacuum-infiltrated (62.5 cm Hg) for 10 min. Explants were then blotted dry on sterile filter paper and cultured on semi-solid MS co-culture medium in Petri plates (100 x 15 mm; 30 mL; up to 50 explants per plate) and cultured in the dark for 2-3 d at 27°C. Hypocotyls were then washed three times with liquid co-culture medium to remove excess bacterial growth, blotted dry, and cultured horizontally on selection-regeneration medium [MS medium supplemented with 22.2 µM BA, 0.5 µM TDZ, 50 mg L^{-1} AS, 10% CW, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin] (15-20 explants per plate) for up to 8 wk. Explants regenerating shoot primordia were moved to selection elongation medium [MS medium with Gamborg et al. (1968) B5 vitamins plus 0.002 g L^{-1} glycine (MSB5G) supplemented with 10 µM BA, 10 µM TDZ, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin] (1–2 explants per vessel). Elongating, kanamycin-resistant shoots were evaluated for the presence of the *nptII* and *cry8D2* genes after four or more subcultures (4 wk interval) on selection elongation medium. Shoots confirmed to contain transgenes were then cultured on multiplication medium [MSB5G medium supplemented with 10 µM BA, 10 µM TDZ, and 500 mg L^{-1} timentin] to increase shoot proliferation rates.

4.3.4 Molecular analysis of transgenic plant lines

Genomic DNA was isolated from leaves of four independent putative-transgenic lines and one control, non-transformed plant following the procedures described by Lefort and

Douglas (1999). A PCR was performed to amplify DNA sequences specific to the *cry8D2* and *nptII* genes. Primers (forward primer *nptII*-F 5'-TGCTCCTGCCGAGAAAGTAT-3' and reverse primer *nptII*-R 5'-AATATCACGGGTAGCCAAGC-3') were designed to amplify a 364 bp PCR product for the *nptII* gene. Primers (forward primer cry8D2-F 5'-AGGGCCCGGATTCGCTACG -3' and reverse primer cry8D2-R 5'-GCCGCTTTGCCTCCTTGACG -3') were designed to amplify a 457 bp PCR product for the *cry8D2* gene. The PCR reaction (25 µL) consisted of 2.5 µL 10x PCR buffer (Invitrogen), 2.5 µL 25 mM Mg^{2+}, 1 µL 50 mM dNTP, 1 µL each of 10 µM *nptII*-F and *nptII*-R or 10 µM cry8D2-F and cry8D2-R primers, 3 µL DNA template (100 ng μL^{-1}), 0.2 µL 5 U μL^{-1} Taq polymerase, and 13.8 µL sterile-deionized water. Plasmid DNA was used as a positive control; DNA from a non-transformed plant and sterile-deionized water served as negative controls. The *nptII* PCR reaction included 2 min at 94°C, followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, 72°C for 2 min, with a final 10 min cycle at 72°C. The *cry8D2* PCR reaction included 2 min at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, with a final 10 min cycle at 72°C. Amplified products of the PCR were visualized under UV light after gel electrophoresis (1% agarose (w/v) plus 0.5 µg mL^{-1} ethidium bromide).

4.4 Results and Discussion

4.4.1 Transformation and regeneration of plants

Six weeks after culture on selection medium, 126 explants out of the 328 not lost to contamination were producing callus or shoot primordia and were moved to selection and elongation medium. Adventitious shoots that elongated in the presence of kanamycin were continuously cultured on selection elongation medium every 4 wk for at least 16 wk. The first 6 weeks of culture on selection and elongation medium initially produced a total of 24 independently elongating, healthy shoots. Four independent transgenic lines were recovered after 8 wk and these shoots grew large enough to remove leaf material for DNA extraction without damaging the microshoots (1.2% transformation efficiency).

Shoot elongation occurred at an extremely slow rate while on medium containing kanamycin, as had been noted for our previous white ash transformation work. Liquid overlays of elongation medium were not attempted to promote white ash shoot growth since these were observed to not be beneficial during our previous study. Once the presence of transgenes was confirmed through PCR, transgenic shoots were cultured on elongation medium without kanamycin. Shoot elongation was still slow after 16 wk on kanamycin-free medium.

Two of the recovered lines elongated individually from the same hypocotyl explant, which continued to maintain healthy shoot primordia throughout 20 wk on selection elongation medium, and elongated several more shoots after 8 wk on elongation medium without kanamycin (shoots were not tested for the presence of transgenes and were not

included in transformation efficiency as these did not elongate within the initial 8 wk culture on selection elongation medium).

Three of the independent lines exhibited normal, healthy growth, and proliferation after 16 wk or more on selection elongation medium, and 24 wk on elongation medium without kanamycin (Fig. 4.2c, d, e). One independent line showed a decline in health during attempts to multiply microshoots (Fig. 4.2b), becoming chlorotic and exhibiting less bud development or elongation after 24 wk on kanamycin-free medium, although it had been green and healthy during 24 wk on selection elongation medium (Fig. 4.2a) and was confirmed through PCR to contain both transgenes (Fig. 4.3; 4.4 lane 4). The aberrant growth of this line could potentially be a result of its prolonged exposure to kanamycin, which was noted to cause chlorosis and death in transgenic *Citrus* microshoots after 3 months of culture (Gutiérrez-E. et al. 1997). The decline in health was not observed until after attempts to multiply the microshoot via nodal sections, which might indicate a negative wounding response. Other factors such as the insertion location of the transgenes within the plant's genome and the expression level of the *cry8D2* protoxin gene could possibly be interfering. Zeng et al. (2010) noted repeated culturing of transgenic white birch resulted in DNA methylation and a decrease in transgene activity.

4.4.2 Analysis of transgenic plant lines

Genomic DNA extracted from the leaves of four putative transgenic lines was used in PCR analysis along with a non-transformed white ash plant. PCR amplification resulted in 457 bp (Fig. 4.3 lanes 4-7) and 364 bp (Fig. 4.4 lanes 4-7) DNA fragments from all four independent lines, corresponding to the *cry8D2* and *nptII* genes, respectively. PCR products of the corresponding size were found in the plasmid control (Fig. 4.3; 4.4 lane

1), but absent in both the non-transformed plant (Fig. 4.3; 4.4 lane 3) and the water control (Fig. 4.3; 4.4 lane 2). This confirmed the successful integration of the foreign genes of interest into *F. americana*. Further work needs to be conducted to confirm the expression of the *cry8D2* gene in these four independent lines.

4.5 Conclusions

Transgenic white ash microshoots containing the *Bacillus thuringiensis* (*Bt*) Cry8Da protoxin gene (*cry8D2*) were successfully developed using *Agrobacterium*-mediated transformation and regeneration protocols for white ash hypocotyl explants. *Agrobacterium* strain EHA105 harboring the pBI121-8D2 vector was used to integrate the *cry8D2* and *nptII* genes into four independent plant lines. Similar methods have been reported for green ash, but only resulted in transgenic callus (Du 2008). Transgene integration was confirmed through PCR analysis of leaf material. This is the first report of regenerating transgenic white ash shoots containing a *Bt* Cry gene. The toxin strain used had previously been shown virulent against EAB adults (Bauer and Lodoño 2011). This work provides a method for obtaining putative EAB-resistant white ash trees through the integration of *Bt* Cry genes. The four independent lines produced in this study are being multiplied for use in future studies. RT-PCR will need to be conducted to confirm the expression of the *cry8D2* gene. Transgenic shoots will eventually be used in a rooting assay and acclimatized to the greenhouse. Plants confirmed to be expressing the toxin gene can be used in future feeding trials with the EAB to determine if the integration of the *cry8D2* gene has potential to impart resistance to attack by the beetle.

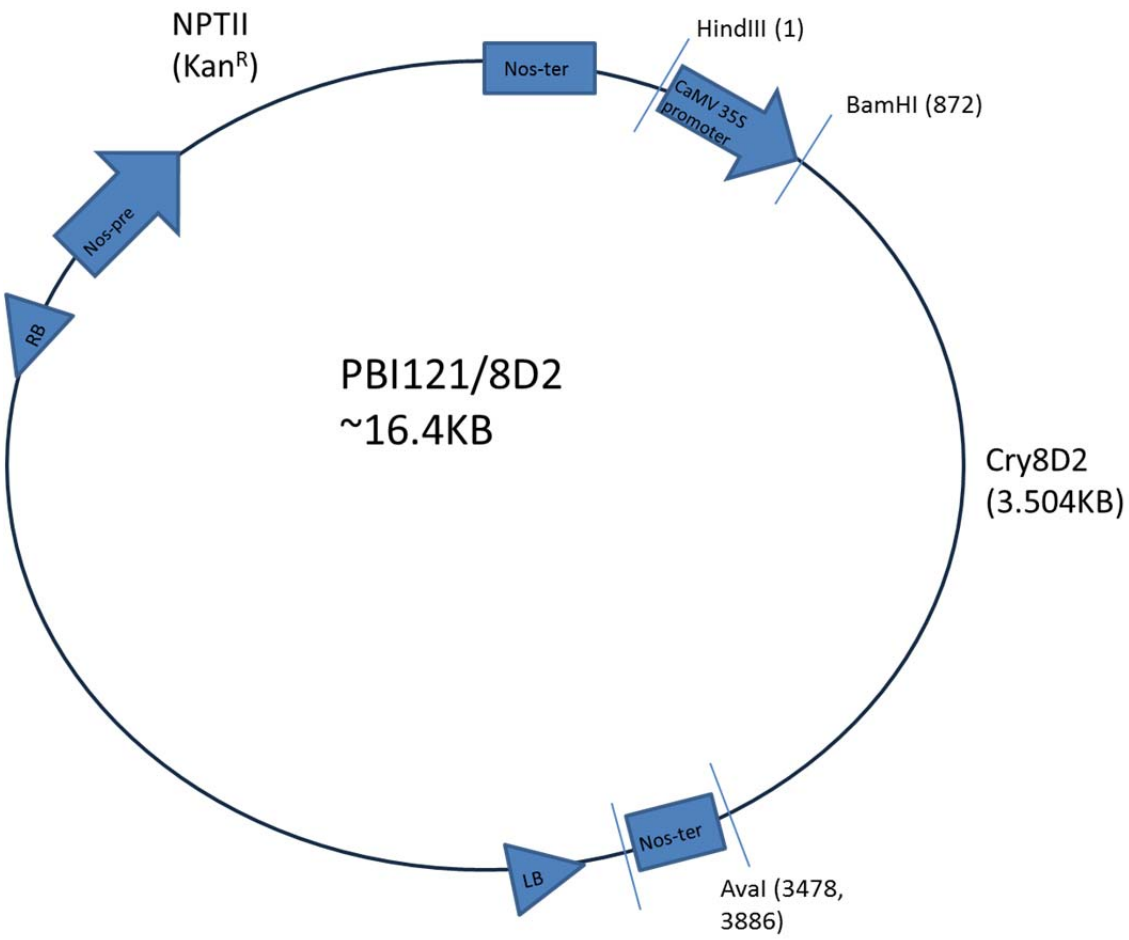

Figure 4.1 The pBI121-8D2 construct. The construct consisted of the pBI121 binary vector, containing the *nptII* gene, the cauliflower mosaic virus (*CaMV*) 35S promoter, *cry8D2* gene, and NOS terminator.

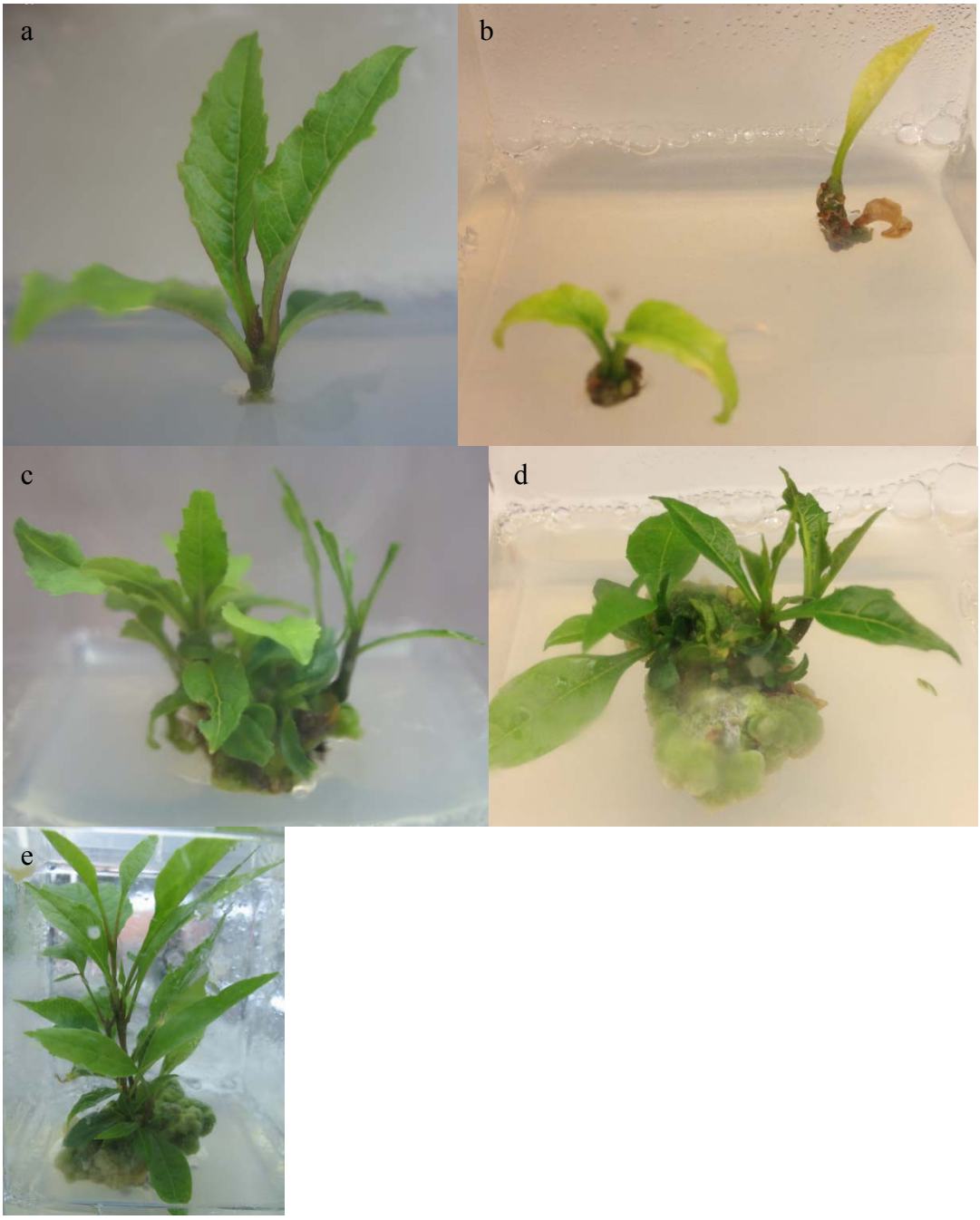

Figure 4.2 Kanamycin-resistant, PCR-confirmed transgenic adventitious Fraxinus americana shoots. (a) Shoot elongating after 20 wk on selection elongation medium [MSB5G plus 10 µM BA, 10 µM TDZ, 30 mg L^{-1} kanamycin, and 500 mg L^{-1} timentin], (b) transgenic shoot during multiplication efforts after 24 wk on elongation medium without kanamycin exhibiting poor growth and chlorosis, (c, d, e) three independent lines of PCR-confirmed transgenic shoots proliferating after 24 wk subcultures on elongation medium without kanamycin exhibiting normal growth.

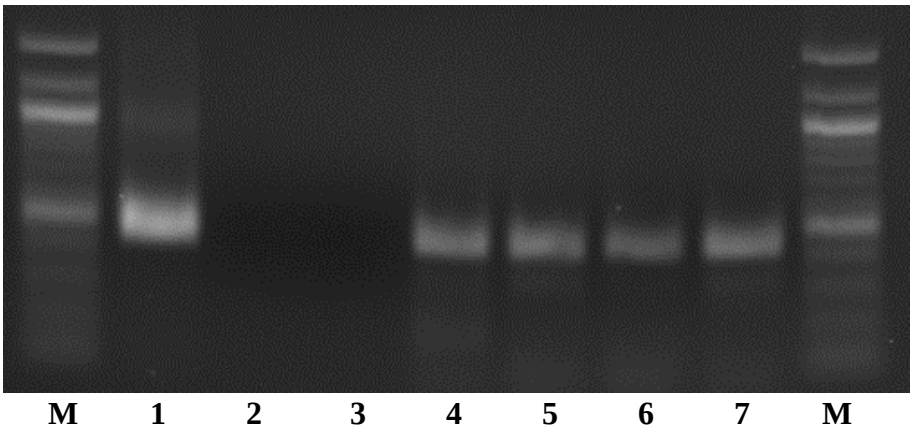

Fig 4.3 PCR analysis of genomic DNA isolated from leaves of non-transformed and transgenic white ash for amplification of 457-bp cry8D2 gene. M 100 bp molecular marker, lane 1 positive control (pBI121-8D2), lane 2 water control, lane 3 negative control (non-transformed plant), lanes 4-7 (putative transgenic shoots), M 100 bp molecular marker.

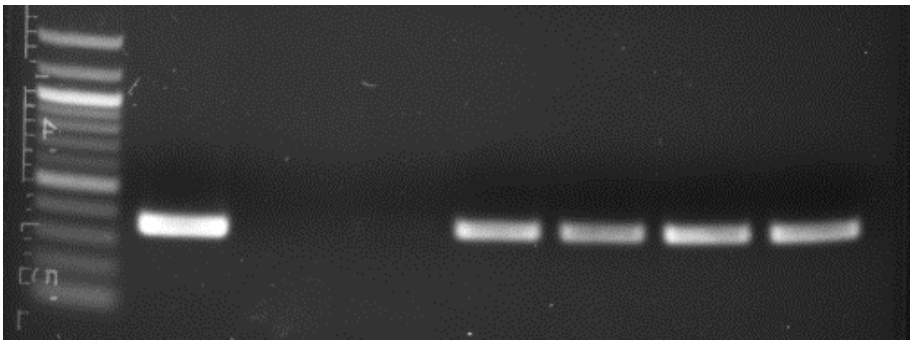

Fig 4.4 PCR analysis of genomic DNA isolated from leaves of non-transformed and transgenic white ash for amplification of 364-bp nptII gene. M 100 bp molecular marker, lane 1 positive control (pBI121-8D2), lane 2 water control, lane 3 negative control (non-transformed plant), lanes 4-7 (putative transgenic shoots).

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